Hypoxia, hypoxia inducible factors and fibrogenesis in chronic liver diseases

CELLULAR AND MOLECULAR MECHANISMS IN LIVER FIBROGENESIS

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Abbreviations used:

ALD, alcoholic liver disease; AFP, alpha-fetoprotein; α-SMA, α-smooth-muscle actin; AMPK, AMP-activated protein kinase; APCs, antigen presenting cells; ASH, alcoholic steato-hepatitis; Atg, autophagy- related gene; bFGF, basic fibroblast growth factor; Bcl2, B-cell lymphoma/leukemia-2; BMP-7, bone morphogenetic protein-7; CCl_4, carbon tetrachloride; CDAA, choline-devoid and aminoacid-refined; CLD, chronic liver diseases; CK-19, cytokeratin 19; CREB, cAMP response element binding protein; CTGF, connective tissue growth factor; DAG, diacylglycerol; DALYs, Disability Adjusted Life Years; DAMPS, damage-associated molecular patterns; DC, dendritic cells; Dhh, Desert hedgehog; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; ER, endoplasmic reticulum; ERK, extracellular signal - regulated kinase; ET-1, endothelin-1; FasL, Fas ligand; FIP200, focal adhesion kinase family-interacting protein of 200 kD; FSP-1, fibroblast-specific protein 1; GFAP, glial fibrillary acidic protein; GFP; green fluorescent protein; Gli, glioblastoma family of transcription factors; GPCRs; G-protein coupled seven-transmembrane receptors; GSK-3β, glycogen synthase kinase-3β; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; Hh, hedgehog; Hhip, Hh interacting proteins; HIF, hypoxia inducible factor; HMGB1, high-mobility group box 1; HPCs, hepatic progenitor cells; HNE, 4-hydroxy-2,3-nonenal; HO-1, heme oxygenase 1; HSC, hepatic stellate cells; HSC-MFs, activated, myofibroblast-like, hepatic stellate cells; HSPs, heat shock proteins; 5-HT, serotonin or 5 hydroxy-triptamine; 5-HTR, serotonin receptor; HVPG, hepatic vein pressure gradient; Ihh, indian hedgehog; IL, interleukin; IL-1R1, IL-1 receptor type 1; IF/MFs, interface myofibroblasts; IFNγ, interferon-γ; IRE1α, inositol requiring protein 1α; JNK1/2, isoforms 1 and 2 of c-Jun-NH2-kinases; LC3, light chain 3; MAPK, mitogen-activated protein kinase; MCP1, monocyte chemoattractant protein 1 or CCL2; MFs, myofibroblasts; MMP, metalloprotease; MET, mesenchymal to epithelial transition; MS, metabolic syndrome; MSC, mesenchymal stem cells; mTOR, mammalian target of rapamycin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-kB, nuclear factor kB; NGF, nerve growth factor; NK, natural killer; NKT, natural killer T; NLR, NOD-like receptor; NLRP3, NOD-like receptor family, pyrin domain containing 3; OLT, ortothopic liver transplantation; PAMPs, pathogen-associated molecular patterns; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMFs, portal myofibroblasts PIIINP, N-terminal peptide of procollagen type III; PBC, primary biliary cirrhosis; PPAR-γ, peroxisome proliferator-activated receptor-γ; PRRs, pattern recognition receptors; PSC, primary sclerosing cholangitis; PS/MFs, portal/septal myofibroblasts; PKA, protein kinase A; Ptc, Patched; RA, retinoic acid; RAE1; retinoic acid inducible gene 1; ROS, reactive oxygen species; SEC, sinusoidal endothelial cells; SERT, specific serotonin transporter; Shh, Sonic hedgehog; Smo, smoothened receptor; TGFβ1, transforming growth factor β1; TGFß2, transforming growth factor β2; THP2, tryptophan-hydroxylase 2; Tie2, angiopoietin I receptor; TIMPs, tissue inhibitor of metalloproteases; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TNFβ, tumor necrosis factor β; TRAIL, TNF-related apoptosis-inducing ligand ULC, Unc-51-like kinase; UPR, unfolded protein response; Vps34, vacuolar protein sorting 34; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor type 2; VHL, von Hippel-Lindau protein; XBP1, X-box binding protein 1.

Abstract

Liver fibrogenesis is a dynamic and highly integrated molecular, tissue and cellular process, potentially reversible, that drives the progression of chronic liver diseases (CLD) towards liver cirrhosis and hepatic failure. Hepatic myofibroblasts (MFs), the pro-fibrogenic effector cells, originate mainly from activation of hepatic stellate cells and portal fibroblasts being characterized by a proliferative and survival attitude. MFs also contract in response to vasoactive agents, sustain angiogenesis and recruit and modulate activity of cells of innate or adaptive immunity. Chronic activation of wound healing and oxidative stress as well as derangement of epithelial-mesenchymal interactions are "major" pro-fibrogenic mechanisms, whatever the etiology. However, literature has outlined a complex network of pro-fibrogenic factors and mediators proposed to modulate CLD progression, with some of them being at present highly debated in the field, including the role of epithelial to mesenchymal transition and Hedgehog signaling pathways. Hypoxia and angiogenesis as well as inflammasomes are recently emerged as ubiquitous pro-inflammatory and pro-fibrogenic determinants whereas adipokines are mostly involved in CLD related to metabolic disturbances (metabolic syndrome and/or obesity and type 2 diabetes). Finally, autophagy as well as natural killer and natural killer - T cells have been recently proposed to significantly affect fibrogenic CLD progression.

Keywords Liver fibrogenesis, hepatic myofibroblasts, chronic liver injury, fibrogenic mechanisms, chronic inflammation, extracellular matrix

Liver fibrogenesis: a driving force for the progression of chronic liver diseases

Definitions, introductory remarks and the overall relevance of fibrogenesis

Chronic liver diseases (CLD) of clinical relevance are typically characterized by persisting parenchymal (i.e., hepatocyte) injury that can be induced by a number of well defined etiological agents or conditions. On a worldwide perspective the following CLD etiologies are the most relevant: i) chronic infection by hepatotropic viruses (hepatitis B and C viruses only); ii) chronic exposure to toxins or drugs (with excess alcohol consumption being predominant in western countries); iii) chronic exposure to altered metabolic conditions; iv) persisting autoimmune injury. Persistent liver injury can result in chronic activation of inflammatory and wound healing response that, in parallel or in association with other pathogenic mechanisms, including at least oxidative stress and the derangement of interactions between epithelial and mesenchymal cells, then sustain liver fibrogenesis, the process that represents a major driving force for liver fibrosis (that is, the net tissue result of fibrogenesis) [1-5].

Accordingly, liver fibrogenesis can be defined as a dynamic and highly integrated molecular, tissue and cellular process that during the course of a CLD leads to a progressive excess accumulation of extracellular matrix (ECM) components (i.e., liver fibrosis) in an attempt to limit the consequences of chronic parenchymal injury [1-3]. Liver fibrogenesis, irrespective of the etiology, is believed to be critical for the progression of any form of chronic liver disease (CLD) and persisting fibrogenesis is widely recognized as the major driving force eventually leading to liver cirrhosis and hepatic failure [1-3,6]. Along these lines, cirrhosis is currently defined as an advanced stage of CLD, characterized by the formation of regenerative nodules of parenchyma surrounded and separated by fibrotic septa, and associated with significant changes in organ vascular architecture, development of portal hypertension and related complications, including variceal bleeding, hepatic encephalopathy, ascites and hepatorenal syndrome [1-6].

In an attempt to describe the relevance of fibrogenesis, according to the concepts nicely outlined in a recent authoritative review on this specific topic [6], one may refer to a general scheme for CLD progression that include at least four stages which intimately related to major pathophysiological events.

The first stage, whatever the etiology, is dominated by the inter-related sequence of persisting chronic parenchymal injury (leading to chronic necrosis and/or apoptosis), chronic inflammatory response and chronic activation of fibrogenesis, which is the driving force for excess deposition of ECM component (i.e., fibrosis). When trying to synthetically describe this first stage (see Figure 1), a number of concepts should be taken in mind [1-6]: i) perpetuation of hepatic injury, a typical hallmark of CLD progression, depends not only from chronic exposure to the specific etiology but also results from chronic injury itself and chronic inflammatory response, through a number of final mediators (with a prevailing role of reactive oxygen species or ROS); ii) chronic activation of inflammatory response and recruitment/activation of cells involved in either innate or acquired immunity can progressively result in what one may define pro-fibrogenic environment, in which synthesis and release of growth factors, cytokines, chemokines, ROS and other mediators will from one side impair significantly hyperplasia/regeneration of hepatic tissue and on the other side will favor chronic activation of wound healing and fibrogenesis; iii) the pro-fibrogenic environment will, in turn, lead to persistent activation of MF-like cells and then increased deposition of ECM components which is paralleled by altered/inefficient remodeling; iv) emerging evidence suggests a major role for hypoxia and angiogenesis in sustaining and, likely, driving fibrogenesis as well as vascular changes that become more and more relevant during CLD progression; v) liver fibrosis in this first stage is potentially reversible, as shown by both experimental and clinical studies; fibrosis reversion may depend on the removal of exposure to the specific etiology or to effective therapy.

When deposition of ECM components becomes significant and fibrotic septa and strictly related vascular changes start to modify significantly the overall structure of liver parenchyma, portal hypertension and related pathophysiological events start to ensue and turn CLD progression into the stage of cirrhosis. Indeed, apart from the histopathological diagnosis of cirrhosis, at least from a clinical point of view, one should not consider cirrhosis as an end point. Rather, it has been suggested the need to define at least two distinct stages of cirrhosis [8]: i) a stage of compensated cirrhosis or cirrhosis without overt clinical manifestations, with hepatic vein pressure gradient (HVPG) still within a range of 5-10 mm Hg; ii) a stage of decompensated cirrhosis or cirrhosis with clinical manifestations (HVPG values > 10-12 mm Hg).

The clinical impact of fibrogenic progression

If persistent liver fibrogenesis may be envisaged as a major driving force for the progression of CLD towards cirrhosis, liver failure and hepatocellular carcinoma (HCC), CLD fibrogenic progression has then a very significant clinical impact which is best described by the following facts [1,6-9].

1. Epidemiological data indicate that approximately 180 millions of patients worldwide are affected by a form of CLD, with HCV chronic infection becoming predominant in western countries, followed by and/or associated with chronic alcohol abuse. Chronic HBV and HCV infections are also predominant in Asia and Africa. 25-30 % of these patients are expected to progress to cirrhosis. In addition, the epidemic of obesity and diabetes will accelerate progression of CLDs and is itself a cause of cirrhosis in the context of evolution of non-alcoholic steatohepatitis (NASH).

2. According to the 2010 Global Burden of Disease study [7], more than one million deaths (representing approx. 2.0% of all deaths) and 31,027,000 Disability Adjusted Life Years (DALYs, that is 1,2% of all DALYs) were due to liver cirrhosis. Alcohol-related liver cirrhosis alone was responsible for 493,000 deaths and 14,544,000 DALYs.

3. Among diseases of the gastro-intestinal tract, cirrhosis represents the most common non-neoplastic cause of death in Europe and USA and overall represents the 7th most common cause of death in western countries.

4. HCC, a very aggressive malignant cancer that represents the 5th most common cancer and the 3rd most common cause of cancer mortality worldwide, almost invariably develops on a cirrhotic background, although initial reports from NASH patients are suggesting that HCC may also develop in a fibrotic liver [8,9]. The annual rate of HCC development in cirrhotic patients has been estimated to vary, depending on etiology, from 2-3% to 7-8% patients.

5. Epidemiologists predict a peak for end-stage CLDs and HCC in the next decade [6,7], in parallel with a shortage of donor organs for ortothopic liver transplantation (OLT), which is currently the only effective treatment option for patients with cirrhosis.

6. Progression of a CLD towards cirrhosis has been estimated to take at least 10 – 15 years and sometimes to require even 30 or more years, but it may be also extremely rapid in particular

clinical settings, such as in children affected by biliary atresia, in patients with HCV recurrence after OLT, or in HCV-HIV co-infected patients [1,6-9]. CLD progression is still then difficult to predict although a number of clinical features have been identified that may serve as predictors for the development of advanced fibrosis and cirrhosis, including: male gender, age <50 years, age at infection (particularly for HCV chronic infection), daily alcohol intake, hepatic iron content, obesity and diabetes mellitus as well as individual factors (differences in immune responses vs infectious agents and related auto-antigens, differences in drug metabolism).

The pattern of fibrosis: the etiology and the fibrogenic cells can make the difference

If cirrhosis can be considered as the common result of progressive fibrogenesis it is now increasingly clear that the specific etiology of a CLD has a relevant impact on CLD progression. According to the view originally proposed in 2004 [10] and then progressively refined in recent years [2,6], a number of defined and distinct patterns of fibrosis development can be recognized in relation to the specific etiology which, in turn, can also significantly influence the prevailing pro-fibrogenic mechanisms and the type(s) of pro-fibrogenic cells involved.

1. Post-necrotic or bridging fibrosis. This pattern is typically observed in the liver of patients affected by chronic viral (HBV, HCV) infection or by an autoimmune disease. The pattern is characterized by the predominant formation of portal-central septa, which follows portal-central bridging necrosis and is associated with interface hepatitis, as well as by the formation of blind septa or septa connecting different portal areas. This pattern then leads to an early involvement of centrilobular vein, formation of neo-vessels (i.e., angiogenesis) and then porto-central shunting. The prevalent mechanism is here represented by chronic activation of wound healing with a contribution of oxidative stress, while pro-fibrogenic cells mainly originate from portal fibroblasts and hepatic stellate cells (HSC).

2. Pericellular fibrosis and capillarization of sinusoids. This pattern, also defined as intercellular fibrosis or chicken-wire fibrosis, is observed in the liver of patients suffering of metabolic derangements such as those with NASH and alcoholic steato-hepatitis (ASH) as well as in patients with hemochromatosis. In these conditions excess deposition of ECM starts in the space of Disse and is mainly the results of activation of perisinusoidal hepatic stellate cells. This results in extensive capillarization of sinusoids which precedes formation of septal bridging and leads to a pattern of fibrosis development that tends to be centro-portal. The prevailing mechanism is here represented by oxidative stress in association to lipotoxicity.

3. Biliary fibrosis. This pattern of fibrosis is seen in primary and secondary biliary cirrhosis as well as in primary sclerosing cholangitis and is characterized by formation of portal-portal fibrotic septa surrounding liver nodules. This pattern of fibrosis develops by preserving connection between central vein and portal tract and is typically associated to intense proliferation of reactive bile ductules and periductular myofibroblasts that are supposed to derive mainly from portal fibroblasts and hepatic stellate cells. Oxidative stress and derangement of the epithelial-stromal equilibrium around bile ducts are believed to play a major role in sustaining fibrogenesis.

4. Centrilobular fibrosis. This is a characteristic pattern of advanced fibrosis which is secondary to venous outflow obstruction (as in heart failure patients). In these conditions the fibrotic septa develop among central vein areas (central-central septa) leading to the unique feature defined as "reversed lobulation".

Liver fibrosis as a potentially reversible process

In the last two decades experimental and clinical studies have provided considerable evidence indicating that liver fibrosis and, possibly, even cirrhosis should be considered as potentially reversible processes [1,3,6,11]. As outlined by several researchers in authoritative reviews [1,3,6,11], critical issues in fibrosis and cirrhosis reversal, providing that the etiological agent or condition may be eliminated or efficiently counteracted by means of selective therapy, are represented by the induction of hepatic MFs apoptosis and by an increased degradation of excess ECM. This has opened the way to experimental studies designed to characterize more selective therapeutic strategies to target either hepatic MFs, specific ligand-receptor interactions or pro-fibrogenic signaling pathways, in order to switch on or at least potentiate fibrosis reversal.

A first relevant issue, described mainly in experimental studies and somewhat confirmed in clinical studies (for example in patients undergoing successful viral eradication), is that cessation of liver injury can result in a significant and progressive regression in the degree of liver fibrosis. As mentioned, the reduction in scar tissue is accompanied by evidence of apoptosis of hepatic myofibroblasts (MFs), with the "rise and fall" of hepatic MFs being modulated by an altered balance between anti-apoptotic and pro-apoptotic signals (coming from either the extracellular or intracellular environment). During CLD progression the feeling is that hepatic MFs may enter into a "survival attitude" that is likely to be the consequence of growth factor- and ROS-mediated activation of Nuclear-Factor kB (NF-kB) [12] and of up-regulation of anti-apoptotic protein bcl-2 [13] in hepatic MFs. Induction of MF apoptosis is supposed to be related to activation of death

receptors in MFs and withdrawal of survival signals. According to this hypothesis, it has been shown that mature nerve growth factor (NGF) and its pro-peptide (proNGF), may act as pro-apoptotic and survival signals, respectively; in particular, during resolution of fibrosis the cleavage of proNGF by MMP-7 altered the proNGF/mature NGF balance facilitating apoptosis of MFs [14].

Apoptosis of HSC/MFs is of course followed by a decrease in collagen production and synthesis of tissue inhibitor of metalloproteases (TIMPs), two major features that favor accumulation of ECM in CLDs. In addition, resolution of liver fibrosis has been reported to be accompanied by a switch in the TIMP/MMP balance, with the mentioned reduction in the hepatic TIMP expression being associated with an increase in the hepatic metalloprotease (MMP) expression [1-5]. A complementary key issue in fibrosis reversal is represented by infiltration of the hepatic scars by bone-marrow – derived macrophages, which are rich source of MMPs that are critical to the successful regression in hepatic scar, with macrophages being then able to promote both liver fibrosis formation and fibrosis regression [1-6,11].

Whether clinical conditions are concerned, several studies have reported evidence suggesting that reversion or regression of fibrosis and cirrhosis may also occur in human patients affected by chronic viral infection (in those patients responding to standard anti-HCV therapy), alcoholic and non-alcoholic steatohepatitis as well as autoimmune hepatitis, following effective therapy and/or removal of etiology [11]. However, as pointed out by different Authors [6,11,15], no incontrovertible evidence has been reported for complete reversal of advanced cirrhosis since a careful analysis of these studies may lead to prudently state that just a variable degree of fibrosis reversion in human patients is described. As discussed by Pinzani and his colleagues [6], this may also be related to a substantial lack of a common language and worldwide consensus on definitions available to unequivocally distinguish conditions of pre-cirrhosis or compensated cirrhosis from true or decompensated cirrhosis, the former being able to show reversal, sometimes significant, the latter being mostly unable to do so.

Cell populations in the scenario of liver fibrogenesis

Whatever the specific etiology or the prevailing pattern of fibrosis, liver fibrogenesis in the scenario of chronic liver injury can be envisaged as a process resulting from intense and persistent interactions (i.e. cross talk) between hepatic cell populations which results from synthesis and release of several mediators (growth factors, cytokines, chemokines, ROS, vasoactive agents, etc). Although, as detailed later, also extrahepatic cells (mainly derived from hemopoietic bone

marrow) may be recruited and involved in chronically damaged liver, practically any resident liver cell population has been reported to play a significant role in CLD fibrogenic progression [1-5]. As summarized in Figure 2, the persisting scenario of etiology-related parenchymal cell injury and death (necrotic and/or apoptotic), chronic inflammatory response and qualitative and quantitative alterations of ECM unavoidably involves activated inflammatory cells, mainly resident Kupffer cells and macrophages recruited from peripheral blood and of bone marrow origin. These macrophages, when activated, offer a major role in CLD progression by synthetizing and releasing a whole battery of established pro-fibrogenic and pro-inflammatory mediators, with platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGFβ1), tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), monocyte chemoattractant protein-1 (MCP-1 or CCL2) and ROS representing the most efficient ones. Liver parenchymal cells (i.e., hepatocytes) are believed to represent a major source of ROS as well as of other oxidative-stress–related reactive mediators or intermediates in addition to be the most significant source, in quantitative terms, of vascular endothelial growth factor (VEGF) during CLD progression. Less obvious but still relevant is the contribution from endothelial cells (damaged/activated) and platelets, which are indeed a significant additional source of growth factors, cytokines and other mediators, with endothelial cells contributing significantly to release of vasoactive agents like NO and entothelin-1. In any form of CLD, however, liver fibrogenesis is mainly sustained by hepatic MFs which represent a heterogeneous population of cells, with the vast majority of them being easily recognized in fibrotic or cirrhotic human liver specimens by the immune-positivity for α-smooth-muscle actin (α-SMA), the most reliable in vivo marker for these cells [16]. Hepatic MFs are indeed major “effectors” of liver fibrogenesis which is remarkably related to their ability to represent (see Figure 2) a unique and critical cellular crossroad able to integrate all incoming paracrine or autocrine signals released from hepatic cell population (growth factors, pro-inflammatory cytokines, chemokines, pro-angiogenic mediators, adipokines, ROS and others).

Hepatic myofibroblasts: origin and phenotypic responses

Different populations of hepatic MFs have been reported to exist in liver specimens obtained from chronically injured liver that may be recognised according to their antigen profile and/or tissue localisation [16]: i) Activated, myofibroblast-like, hepatic stellate cells (HSC-MFs), which are αSMA-positive cells found primarily in or around capillarised sinusoids of fibrotic/cirrhotic livers, which originate mainly from activation/transdifferentiation of hepatic stellate cells (HSC); ii) Portal/septal MFs (PS/MFs), which can be found in the expanded connective

tissue around portal tracts (portal MFs) or in the inner part of fibrotic septa (septal MFs); portal and septal MFs, which display an overlapping antigen repertoire, originate from portal fibroblasts (PFs) through a process of activation/transdifferentiation; iii) Interface MFs (IF/MFs), that are found at the edge between fibrotic septa and the surrounding parenchyma (i.e., where active fibrogenesis occurs) that are believed to likely originate from all the different cellular sources hypothesized in these years [1-5].

Apart from the tissue localization, the heterogeneous population of hepatic MFs can then originate mainly from HSC or portal fibroblasts through a process of activation/trans-differentiation. This process leads to a highly proliferative, migratory and contractile MF-like phenotype, able not only to synthetize excess ECM components and to remodel the matrix itself but also to act in order to sustain angiogenesis and/or inflammatory response as well as to modulate immune response. Indeed, most of actual knowledge on hepatic MFs (Figures 3 and 4) comes directly from studies performed on HSC/MFs but the feeling of researchers involved in the field is that the most relevant phenotypic responses of MF-like cells are likely to be common to MFs originated from portal fibroblasts and, possibly, from other sources. Accordingly, hepatic MFs, although to a less extent, have been also reported to originate from cells derived from bone marrow (mesenchymal stem cells or MSC, fibrocytes or even hemopoietic stem cells) and recruited/activated in the chronically injured liver parenchyma [1-5]. Literature data also support the concept that at least part of pro-fibrogenic cells in CLD may originate through a process of epithelial to mesenchymal transition (EMT) involving either hepatocytes or cholangiocytes [17], an issue that is actually the subject of intense debate.

Established Pro-fibrogenic mechanisms

According to the original proposal by Pinzani and Rombouts [10], as refined in the following years [2,6,17,18] three prevailing mechanisms have been involved in liver fibrogenesis, including: i) chronic activation of wound healing, ii) oxidative stress and iii) derangement of epithelial-mesenchymal interactions.

Chronic activation of wound healing

Chronic activation of wound healing in CLD and of hepatic MFs, whatever the etiology, is believed to represent the most common and relevant mechanism sustaining hepatic fibrogenesis, as suggested by the following issues:

- the persistence of chronic liver injury (hepatocellular and cholangiocellular) resulting in a variable degree of necrosis and/or apoptosis;

- the persistence of an inflammatory infiltrate mainly composed of macrophages and lymphocytes;

- the persistent involvement of pro-fibrogenic, MF-like (i.e., α-SMA-positive), ECM-producing cells characterized by high proliferative attitude, migration and contractility as well as by all other phenotypic responses summarized in Figure 3;

- the detection of excess (i.e. quantitative) deposition and qualitative alteration in the composition of the ECM, the latter being associated with an impaired ability to remodel/remove fibrillar-like collagens and a persistent attempt to repopulate liver parenchyma (i.e., liver regeneration or compensatory hyperplasia).

Chronic activation of wound healing reaction and hepatic MFs is believed to be sustained by several growth factors and cytokines, chemokines, ROS and other reactive intermediates from oxidative stress as well as by other established and continuously emerging polypeptides. According to in vivo and in vitro studies several laboratories have dissected pro-fibrogenic receptor-mediated or receptor-independent intracellular signalingpathways elicited by these mediators. Polypeptide factors, in particular, include at least PDGF, TGFß1, connective tissue growth factor (CTGF), endothelin-1 (ET-1), MCP1 or CCL2, TNFα, adipokines and pro-angiogenic factors (including VEGF-A and Angiopoietin I) [1-5].

Oxidative stress

Involvement of oxidative stress, reactive oxygen species (ROS) and other reactive intermediates has been unequivocally documented in most experimental models of liver fibrogenesis and in all human major clinical conditions of CLDs. Oxidative stress, in particular, has been reported to play a predominant pro-fibrogenic role in patients affected by non-alcoholic and alcoholic steatohepatitis (NASH and ASH patients) [1-5,18]. The following major established concepts should be briefly recalled.

1. Oxidative stress in CLDs results from either increased generation of ROS and other reactive intermediates, due to the specific impact of the etiology, by injured parenchymal cells (i.e., hepatocytes) and/or activated inflammatory cells. For example, ROS generation may follow altered metabolic state (like in non-alcoholic fatty liver disease or NAFLD and NASH) or ethanol

metabolism (ASH), with ROS being then mainly generated by mitochondrial electron transport chain or through the involvement of selected cytochrome P450 isoforms like CYP2E1 or other redox enzymes. This scenario is often paralleled in progressive CLDs by a consistent decrease in the efficiency of antioxidant defences. Moreover, in conditions of severe oxidative stress ROS and other intermediates, like aldehydic end-product of lipid peroxidation 4-hydroxy-2,3-nonenal (HNE) can be released by either activated inflammatory cells or from injured hepatocytes to significantly contribute to perpetuation of cell death [1-5].

2. ROS, HNE and other related reactive mediators released by damaged hepatocytes or activated inflammatory cells have been reported to unequivocally affect/modulate the behaviour of human HSC/MFs and likely of other MF-like cells. Along these lines, ROS and HNE have been reported to up-regulate expression of critical pro-fibrogenic genes, including pro-collagen type I, TIMP-1 and MCP-1 (CCL2), possibly through activation of specific signal transduction pathways and transcription factors, including activation of JNKs, AP-1 and, only for ROS, NF-kB [1-5,18]. Similarly, intracellular ROS generation by NADPH-oxidase in HSC/MFs has been reported to sustain pro-fibrogenic signaling pathways in response to pro-fibrogenic mediators, including PDGF-BB, angiotensin II, and the adipokine leptin [19]. Accordingly, selective inhibition or genetic manipulation of NADPH oxidase subunit can effectively reduce either phenotypic responses of HSC/MFs or fibrogenesis (reviewed in [19]).

3. ROS, not HNE, have been reported to stimulate proliferation of HSC/MFs and ROS can also contribute to stimulate oriented migration of these pro-fibrogenic cells by involving activation of isoforms 1 and 2 of c-Jun-NH2-kinases (JNK1/2). This redox-sensitive pro-migratory action relies just on a significant increase of intracellular ROS, whatever their origin; HSC/MFs can migrate in response to i) ROS entering them from the chronically injured microenvironment, ii) ROS released by mitochondria following exposure to hypoxia or iii) ROS generated by NADPH-oxidase activation that parallels the activation of ligand-receptor interaction elicited by chemotactic polypeptide factors such as PDGF-BB, VEGF-A or MCP-1 [20,21].

4. Experimental studies from ethanol-induced chronic injury in rodents and clinical data from patients affected by alcoholic liver disease (ALD), chronic HCV infection or NAFLD indicate that oxidative stress- mediated injury can result in the development of circulating IgG antibodies directed against epitopes derived from protein modified by ROS or other intermediates. Titre of

these antibodies correlate with disease severity and, as proposed for either ALD or NAFLD patients, may serve as a prognostic predictor of progression of CLD to advanced fibrosis [22].

Derangement of epithelial/mesenchymal interactions

This particular mechanism [2,3,6,10] is involved mainly in cholangiopathies, a group of progressive disorders representing a major cause of chronic cholestasis in adult and pediatric patients. These disorders share a common scenario characterized by cholestasis, necrotic or apoptotic loss of cholangiocytes, cholangiocyte proliferation and portal/periportal inflammation and fibrosis. These disorders are characterized at histopathological level by the so called “ductular reaction”, consisting in an intense proliferation of cholangiocytes that is associated with significant changes in the surrounding mesenchymal cells. These changes involve first portal fibroblasts in the portal connective tissue and then HSC when the reaction starts to invade the surrounding parenchyma, and are also associated with significant changes in the ECM. An intense cross-talk between cholangiocytes and surrounding mesenchymal cells is believed to result in the release of cytokines and pro-inflammatory mediators that are responsible for the overall scenario described for cholangiopathies. Injured/activated cholangiocytes are considered active “cellular actors” for their ability to secrete several chemokines (IL-6, tumor necrosis factor β or TNFß, IL-8, MCP-1) and pro-fibrogenic polypeptide mediators (PDGF-BB, ET-1, CTGF, transforming growth factor β2 or TGFß2). The hypothesis here is that these factors released by activated/injured cholangiocytes are responsible for initiation of myofibroblastic differentiation of portal fibroblasts that express all related receptors. Similarly to what is known to happen during HSC trans-differentiation into HSC/MFs, this event is believed to be followed by an autocrine perpetuation of portal MFs activation resulting in biliary-like fibrosis. This putative pro-fibrogenic scenario is also sustained by the fact that almost the same chemokines and pro-fibrogenic polypeptides can also be produced by infiltrating immune, inflammatory or mesenchymal cells [2,3,10]. Moreover, portal MFs are increasingly recognized as cells able to contribute to fibrogenic CLD progression in other clinical conditions of different etiology and characterized by bridging fibrosis. Indeed, what is emerging to be relevant is not the specific etiology but the cross-talk between damaged/activated cholangiocytes and portal fibroblasts and the differentiation of the latter into portal MFs, and indeed ductular reaction has been reported also in HCV chronic patients as well as in NASH patients [3]. This concept has also a relevant pathophysiological implication, as recently shown by a very elegant study which has identified the Notch signalling as the one governing the ability of

hepatic progenitor cells (HPC, which are in portal tracts at the level of Hering's duct) to differentiate into either hepatocyte or cholangiocyte [23]. If the cellular targets of chronic injury are cholangiocytes their interaction with surrounding cells leads MFs in the area to generate Jagged-1 which, in turn, drives differentiation of HPC towards cholangiocyte, also contributing to sustain ductular reaction. If the targets of injury are predominantly represented by hepatocytes, macrophages or Kupffer cells release Wnt3a that switches off Notch signaling in HPC leading them to hepatocellular differentiation.

Emerging and/or debated mechanisms in liver fibrogenesis

In the last decade several novel mediators and mechanisms, as well as entire signaling pathways and systems, have been proposed to play an active and/or critical role in sustaining liver fibrogenesis during the progression of CLDs. In this review we have focused the attention on a defined number of emerging aspects and issues rather than to attempt to describe and discuss any possible "mechanism" claimed to be relevant.

Epithelial to mesenchymal transition (EMT) process and liver fibrogenesis

Epithelial to mesenchymal transition or EMT refers to a fundamental biologic process in which cells of epithelial origin undergo a phenotypic and functional transition towards cells with mesenchymal properties. In particular, epithelial cells undergoing EMT lose their polarization and specialized junctional structures, undergo cytoskeleton reorganization, and ~~to~~ acquire morphological and functional features of mesenchymal-like cells, including the ability to migrate and to produce and secrete components of the extracellular matrix [23-24]. EMT and the related opposite process of MET (mesenchymal to epithelial transition) have been originally described in embryonic development (where cell migration and tissue remodeling have a primary role in regulating morphogenesis). More recent literature data have provided evidence suggesting that the EMT process may also have a significant role in cancer progression and also organ fibrosis [23-24]. In particular, EMT has been proposed to contribute to fibrosis in those chronic conditions characterized by uncontrolled activation of wound-healing response and progressive fibrogenesis, then including also CLD. Pertinent to this review, several studies [reviewed in 17, 24], have proposed that pro-fibrogenic cells, in addition to the cellular sources already mentioned, may originate in CLD through EMT involving either cholangiocytes or hepatocytes.

Following homologous studies on kidney and lung fibrosis, the first liver-related experiments showed that hepatocytes or cholangiocytes cultured in the presence of TGF-β rapidly acquire a spindle-like or fibroblastoid-like morphology accompanied by canonical EMT related changes, including E-cadherin down-regulation and up-regulation of mesenchymal markers like vimentin, desmin, α-SMA and the protein S100A4, the latter also termed FSP-1 (fibroblast-specific protein 1). In the first of these studies [25] AlbCre.R26RstoplacZ double transgenic mice were used to investigate whether hepatocytes undergoing EMT may contribute significantly to fibrosis in the chronic carbon tetrachloride (CCl_4) model. Authors reported that approx. 15% of hepatic cells were FSP-1 positive at the time of severe fibrosis and that approx. 5% of the hepatic cells were co-expressing either FSP-1 and albumin or FSP-1 and β-gal, then suggestive of EMT. Moreover, in the same study the treatment with bone morphogenetic protein-7 (BMP-7), which is known to antagonize TGFβ1 signaling, significantly inhibited liver fibrosis and almost abolished putative EMT-derived fibroblasts/MFs, a result also obtained by another laboratory using transgenic mouse over-expressing Smad7 in hepatocytes [26]. The latter study also reported some morphological evidence for “in vivo” EMT in biopsies from chronic HBV patients.

Experimental and clinical studies also suggested involvement of EMT of cholangiocytes in biliary-like fibrosis, with cholangiocytes under these conditions apparently co-expressing α-SMA and cytokeratin 19 (the latter being a marker for cholangiocytes and hepatic progenitor cells or HPCs) [27]. A very close scenario was confirmed in the same model of BDL (rat and murine) by a series of elegant studies from the group of Anna Mae Diehl [17], the most relevant being able to describe an apparently clear cause-effect relationships among EMT of cholangiocytes, appearance of portal MFs and biliary fibrosis as well as the closely related major involvement of Hedgehog signaling pathway. Morphological evidence for EMT of cholangiocytes was also described in liver biopsies from human patients affected by primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) or biliary atresia [17,24]. In addition, the relevance of Hedgehog and TGFβ1-Smad2/3 signaling was reported also in human patients [17,24]. More recently, Notch signalinghas been also implicated in EMT and liver fibrosis based on the reported action of the γ-secretase inhibitor DAPT that, by inhibiting Notch signaling, blocked an EMT-like transition in both primary mouse HSCs and a rat HSC cell line as well as inhibited HSC activation and attenuated CCl_4-induced rat liver fibrosis [28,29]. Similar reports suggested the efficacy in blocking cholangiocyte EMT by using the same γ-secretase inhibitor or a Jagged1-neutralizing antibody [29, 30].

The initial enthusiasm has been tempered by a number of very elegant studies that have seriously challenged the involvement of EMT of either hepatocytes or cholangiocytes as major pathogenic mechanism in liver fibrogenesis. In a first study Authors employed triple transgenic mice expressing ROSA26 stop beta-galactosidase (beta-gal), albumin Cre, and collagen alpha1(I) green fluorescent protein (GFP), in order to have hepatocyte-derived cells permanently labelled by beta-gal and type I collagen-expressing cells labelled by GFP [31]. Using this approach in either in vitro conditions or in vivo models of fibrosis, Authors could not find cells expressing double-positivity for GFP and beta-gal. Moreover, all beta-gal-positive cells exhibited the typical morphology of hepatocytes and did not express mesenchymal markers like α-SMA, FSP-1, desmin, or vimentin. Even more relevant, GFP-positive areas in fibrotic livers were coincident with fibrotic septa but never overlapped with X-gal-positive areas, suggesting that type I collagen-producing cells were not originating from hepatocytes.

A very similar conclusion was reached in a second study from the same group [32] in which EMT was again investigated with Cre/LoxP system in order to map cell fate cytokeratin 19 (CK-19) positive cholangiocytes in CK-19(YFP) or FSP-1(YFP) transgenic mice that were then subjected to bile duct ligation or chronic carbon tetrachloride treatment. When the livers of fibrotic transgenic mice were analyzed by specific immunostaining of CK-19(YFP) cholangiocytes showed no expression of EMT markers such as α-SMA, desmin, or FSP-1. Moreover, cells genetically labelled by FSP-1(YFP) expression did not co-express neither the cholangiocyte marker CK-19 nor E-cadherin. These results led again Authors to conclude that EMT of cholangiocytes was not contributing to liver fibrogenesis in these murine models.

A third study by the group of Brenner provided compelling evidence that FSP-1 (the putative and widely used marker for EMT-derived fibroblasts) in either human and experimental CLDs was indeed not expressed by HSC or type I collagen-producing fibroblasts [33]. Moreover, FSP1-positive cells did not express classical markers of MFs like α-SMA and desmin, and were not precursors of MFs in injured livers as evaluated by genetic lineage tracing experiments. Surprisingly, FSP1-positive cells expressed F4/80 and other markers typical of the myeloid-monocytic lineage and the overall data indicated that FSP1 was expressed by a subset of macrophages involved in CLD progression that differed from Kupffer cells.

A fourth study challenging EMT [34] employed lineage tracing generated by crossing the alpha-fetoprotein (AFP) Cre mouse with the ROSA26YFP stop mouse in order to trace the fate of any cell

ever expressing AFP. This approach led to a genetic labeling of all cholangiocytes and hepatocytes, because all these epithelial cells derived from AFP-expressing precursor cells. The critical result here was that after inducing liver fibrosis using different models, none of the resulting MFs was found to originate from the genetically marked epithelial (AFP+) cells. An almost identical conclusion was reached by a fifth study which reported that Vimentin-CreER marked MFs did not undergo mesenchymal to epithelial transition [35].

The real involvement of EMT as a pathogenic mechanism contributing to liver fibrogenesis in CLDs is then more than controversial, with accumulating evidence deposing against EMT from either hepatocytes or cholangiocytes [31-36]. Along these lines, an excellent very recent fate tracing study from the laboratory of Robert Schwabe, performed using a novel Cre-transgenic mouse that marks 99% of hepatic stellate cells, has shown that HSCs give rise to 82-96% of myofibroblasts in models of toxic, cholestatic and fatty liver disease [37]. This study unavoidably strongly suggests that any contribution by EMT to fibrogenesis should be considered as minor.

This highly debated concept has been recently reviewed in an exhaustive manner by leading researchers in the field [38] that described evidence for and against the involvement of EMT in liver fibrogenesis. Authors of this review also present most recent data obtained in their laboratory using again very sophisticated transgenic mice; these latter results still seem to support a role for EMT, although they point to one even more critical and somewhat unexpected concept: HSCs in the liver may represent a resident population of inherently plastic cells which can be reprogrammed by mesenchymal to epithelial transition (MET)-EMT to replace adult liver epithelial cells.

Hedgehog signaling in the fibrogenic arena

Hedgehog (Hh) signaling pathway is known to regulate critical cellular responses including proliferation, apoptosis, migration, and differentiation. The Hh signaling pathway has been reported to play a critical role in tissue morphogenesis during fetal development. It also modulates wound healing responses in a number of adult tissues and organs, including the liver during progression of CLDs [39]. Canonical Hh signaling is initiated by the interaction of a family of ligands (Sonic hedgehog – Shh, Indian hedgehog – Ihh, and Desert hedgehog – Dhh) which interact with the Patched (Ptc) specific cell surface receptor that is expressed on the plasma membrane of responsive cell. These ligands can be released by several cells exposed to specific stimuli and relevant examples are represented by Shh ligand, released by HSC stimulated with PDGF or leptin;

similarly, Shh and Ihh ligands have been reported to be released by hepatocytes undergoing caspase 3-dependent apoptosis induced by several triggers, including TGFβ. However, TGFβ has been reported to also activate the signaling in a Ptc and Smoothened (Smo) independent way (non-canonical pathway) by eliciting increased transcription of glioblastoma (Gli) 2 [40]. The signaling can be initiated via autocrine, paracrine or endocrine loops since the source of Hh ligands may be a Hh-responsive cells itself or a neighboring cells or even a distant cells releasing Hh ligands in membrane-associated particles (exosome-like).

Ligand-receptor interaction de-represses activity of another molecule, Smoothened (Smo), responsible for the propagation of the signal that leads to nuclear translocation of members of Glioblastoma (Gli1, Gli2, Gli3) family transcription factors, that regulate the expression of a number of critical Gli-target genes. In the absence of Hh ligands, Ptc represses Smo and leads to Gli ubiquitination and subsequent proteasomal degradation. Nuclear translocation of Gli transcription factors is modulated by other factors [41], including insulin-like growth factor that inhibits protein kinase A (PKA)-dependent phosphorylation of Gli1 in Hh-responsive cells, leading then to inhibition of Gli phosphorylation by glycogen synthase kinase-3β (GSK-3β) which, in turn, prevents its proteosomal degradation. On the other hand, Hh–related transcription factors Gli can regulate the transcription of pleiotropic TGFβ-target genes, such as Snail1 [42], and influence expression of factors that are able to modulate Wnt signaling, including Wnt5a (a Wnt pathway activator) and soluble frizzled receptor-1, an inhibitor of Wnt signaling [43].

Experimental and clinical studies that have investigated the role of Hh ligands and signaling pathway in normal and diseased liver have pointed out a number of relevant issues. As a first notion, differently from developing liver, normal liver usually expresses quite low levels of Hh ligands but, in turn, sinusoidal cells (HSC and sinusoidal-endothelial cells or SEC) express relatively high levels of Hh interacting proteins or Hhip, which are known to bind Hh ligands and then to prevent their interactions with respective receptors. Moreover, Hh signaling pathway has been reported to be progressively silenced during the process of liver epithelial cell maturation, with Ptc expression being highly expressed in bipotent HPCs but very low in adult hepatocytes [44].

By contrast, in the progression of CLD most of liver resident cell populations (hepatocytes, cholangiocytes, SEC, HSC/MFs and natural-killer T (NKT) cells) have been shown to produce and release these ligands when exposed either to cytotoxic or pro-apoptotic stress as well as to relevant growth factors and cytokines generated during liver fibrogenesis. Under toxic or stress

conditions production and release of Hh ligands increases concomitantly to repression of Hhip expression by activated HSC or SEC; this allows ligand-receptor interaction and activation of the Hh signaling pathway in Hh-responsive cells, including NKT cells, cholangiocytes, progenitors and quiescent hepatic stellate cells. Increased hepatic expression of Hh ligands and their accumulation in chronically injured liver indeed parallels the increase in the number of Hh-responsive cells according to the severity of liver injury and the extent of fibrosis [39].

The activation in a paracrine way of Hh signaling in each of these target cells has been reported to contribute and/or sustain liver fibrogenesis. Indeed, several experimental studies (using transgenic mice and/or inhibitors of Hh signaling such as Smo antagonists) and clinical studies, have outlined a number of mechanisms, summarized as follows also in relation to the specific target cell:

1. Hh ligands have been reported to directly and significantly contribute to the process of HSC activation and trans-differentiation, helping then to drive quiescent HSC towards the activated- and MF-like (i.e., pro-fibrogenic) phenotype, with HSC/MFs being both a source of and a target for these ligands. Hh ligands, in particular, mainly sustain proliferation of these cells as well as their ability to synthesize and release ECM components [44,45].

2. Hh ligands can promote cholangiocyte proliferation, favoring the so-called ductular reaction which is typical in cholangiopathies and in the biliary fibrosis pattern. Moreover, Hh ligands can also act on cholangiocytes by up-regulating their expression of chemokines like CXCL16, resulting in a related recruitment of subpopulations of Hh ligand producing- and Hh-responsive immune cells and inflammatory cells in the injured liver (e.g., NKT cells) into liver [46-48]. In particular, Hh ligands and activation of Hh signaling pathway can eventually stimulate NKT cells to produce other putative fibrogenic factors like IL-4 and IL-13 [47,48].

3. The expansion of the cells able to respond to Hh ligands (for example HSC/MFs population) practically also results in an increase of those critical pro-fibrogenic factors produced in an autocrine-paracrine loop by these cells such as mainly PDGF and TGFβ that, in addition to their well-known pro-fibrogenic potential, can exacerbate Hh signaling pathway-related events by stimulating further production of Hh ligands [39].

4. Hh ligands and Hh signaling pathway have been reported as relevant in sustaining either hepatocyte or cholangiocyte EMT, although this issue is still debated (see the previous section), as well as to promote epithelial to mesenchymal transition in hepatic stellate cells [39].

5. Increased expression of Hh ligands in condition of liver injury have been reported to activate liver sinusoidal endothelial cells, causing them to express adhesion factors and other mediators that contribute to vascular remodeling, a relevant event in the progression of CLD.

As nicely summarized in a recent review on this topic [39], the overall message, in relation to the potential role of Hh pathway in liver pathophysiology, is that although transient activation of this signaling pathway is somewhat necessary for adult livers to regenerate after an acute injury, the sustained increase in Hh signaling occurring in the presence of persistent injury can perpetuate the expansion and the pro-fibrogenic activity of critical cell types, mainly HSC/MFs.

Adipokines

Adipokines represent a group of polypeptide hormones which are expressed predominantly by adipocytes and also by cells of the stroma and macrophages. Interest in these cytokines comes from the evidence that their expression in terms of blood levels is significantly modulated in patients which are either obese and/or affected by metabolic alterations resulting in insulin-resistance [49]. In particular, obesity and insulin resistance have been shown to correlate with fibrogenic progression in CLDs [49,50]; moreover, accumulation of white adipose tissue or type 2 diabetes, as features of the so-called metabolic syndrome (MS), are considered as significant risk factors for the progression of NASH to cirrhosis. Along these lines, a consistent bulk of recent literature data indicates that different member of the adipokine family, including at least leptin, adiponectin, resistin and a few emerging other adipokines, can be considered as effective modulators of liver fibrogenesis.

Leptin. Leptin, the best characterize adipokine, is the product of the obese (ob) gene and is mainly expressed by adipose tissue, although other tissues may contribute to its secretion [49]. Leptin is known to act by limiting food intake and, accordingly, obesity in humans has been associated with the absence of leptin or with a scenario of reduced leptin-mediated signaling. The pro-fibrogenic role of leptin has unequivocally emerged from several studies performed either in rodent models of fibrosis or in cultured cells. In vivo studies have established that both the absence of leptin or

an impairment of leptin signaling can almost abolish the development of in vivo experimental fibrosis [51]. The effect of leptin is direct since food restriction does not restore fibrogenesis and because injection of recombinant leptin has been shown to accelerate fibrogenesis. In particular, leptin can indeed directly act on HSC, Kupffer cells as well as sinusoidal endothelial cells. HSC have been shown to express ObRb leptin receptor, the isoform that is known to display full signaling capabilities [52]; ObRb expression is low in quiescent HSC to become then up-regulated during the process of activation/trans-differentiation towards the activated, MF-like phenotype [53]. Moreover, leptin has been shown to up-regulate type I procollagen expression, to potentiate TGFβ1-mediated effects [53], to induce tissue inhibitor of metalloproteinase-1 (TIMP1) expression [54] as well as to stimulate HSC proliferation and survival [55], and up-regulate expression of chemokines (like CCL2) in a NF-kB-dependent way [52]. Although leptin may stimulate multiple signaling pathways, literature data suggest that its pro-fibrogenic effects are mainly NADPH oxidase and ROS dependent [56] and may also be mediated by inhibition of the expression and activity of peroxisome proliferator-activated receptor-γ (PPAR-γ), which maintains HSC quiescence and reverses HSC trans-differentiation to MFs. Recently, leptin has also been shown to modulate liver angiogenesis being able to both exert a direct angiogenic action in vascular endothelial cells [57] and stimulate HSC to increase expression and release of vascular endothelial growth factor A (VEGF-A), which is the most potent pro-angiogenic cytokine [52]. This is relevant because of the emerging evidence indicating that angiogenesis is not only a critical component of chronic wound healing but can indeed favor liver fibrogenesis. Finally, it has also been reported that leptin, in addition to stimulate increased phagocytic activity and cytokine secretion by Kupffer cells and macrophages [58], may act indirectly on HSC by stimulating Kupffer cells to up-regulate their expression of TGFβ1 [59].

Adiponectin. Adiponectin is one of the most abundant plasma proteins and its circulating levels are inversely correlated with fat mass, and directly correlate with insulin sensitivity [60]. Adiponectin act in cells expressing AdipoR1 and AdipoR2 receptors, which are predominantly expressed in skeletal muscle and the liver, respectively. Literature data indicate that adiponectin has predominantly hepato-protective and anti-fibrogenic effects in conditions of CLD [61], as shown in particular in the experimental model of fibrosis induced by chronic administration of CCl_4 [62]. Adiponectin has likely a protective role also in the context of NAFLD/NASH as shown by experiments in which pericellular fibrosis was found to be more severe in adiponectin-deficient mice (i.v., vs wild type mice) fed a high-fat diet [63]. Moreover, adiponectin has been reported to

delay the progression of experimental NASH towards cirrhosis and hepatocellular carcinoma [64]. Adiponectin has been also proposed to act as a negative modulator of inflammation [65], an observation that becomes relevant in the scenario of adipose tissue inflammation, which is known to lead to release of free fatty acids and to an altered pattern of adipokine secretion [66]. However, this anti-inflammatory action of adiponectin is currently debated. In any case, adiponectin can act directly on HSC because these cells express both types of specific receptors [67,68]. Adiponectin has been reported to result in a suppression of both cytokine-stimulated proliferation and migration [62,67], to reduce TGFβ1-dependent effects [62] and even to favor induction of HSC apoptosis [68]. These anti-fibrogenic effects of adiponectin are likely to be mainly mediated through activation of AMP-activated protein kinase (AMPK), a critical downstream effector of AdipoR1 [67], although a single report suggested that blocking AdipoR2 expression worsened the development of fibrosis in a mouse model of dietary steatohepatitis [69].

Resistin. The biological role of resistin in humans is still controversial [49] and seems to differ for several aspects from what described in rodents. Indeed, in rodents resistin is up-regulated in diet-induced or genetic obesity [70] whereas in humans seems to have a role in sustaining inflammation, as shown by studies in which resistin is up-regulated in monocytes exposed to pro-inflammatory stimuli and in conditions of experimentally induced endotoxemia [71,72]. In relation to liver fibrogenesis, it has been shown that resistin expression during liver injury positively correlates with infiltration of inflammatory cells, which may represent the principal source of intrahepatic resistin [73]. Indeed, resistin expression was found to co-localize with the inflammatory cell marker CD43 in liver specimens from patients with alcoholic hepatitis. However, resistin is apparently expressed only in quiescent HSCs isolated from rats, but not in activated HSCs isolated from either rodents or humans [73]. Human HSC, however, respond to recombinant resistin by up-regulating expression of CCL2 and IL-8 in a NF-kB-dependent manner [73].

Apelin. This adipokine represent ~~the~~ endogenous ligand of the orphan G protein-coupled receptor APJ, and is known to display a pro-angiogenic role [74]. The interest in apelin, which is expressed and secreted by adipose tissue both in mice and humans [75], has been generated by a study reporting that its levels were markedly increased in cirrhotic liver [76]. Apelin was found to be highly expressed by HSCs and in the same study the use of an antagonist of the apelin receptor was reported to inhibit not only angiogenesis but also hepatic fibrosis. In addition, exposure of LX-2, a human HSC line, to recombinant apelin resulted in an increased synthesis of type I collagen as

well as of PDGF-β receptor [77]. Moreover, available data on LX2 cells at present suggest that apelin expression can be up-regulated by angiotensin II and endothelin-1 [77] whereas expression of the apelin receptor APJ is positively stimulated by exposure to hypoxia and lipopolysaccharide [78].

Hypoxia and angiogenesis

Angiogenesis can be defined as a dynamic, hypoxia-stimulated and growth factor-dependent process that can occur in any vascularized tissue or organ resulting in the formation of new blood vessels from pre-existing ones in order to ensure an adequate delivery of oxygen and nutrients [79-80]. Although angiogenesis may be considered as beneficial for tissue growth and regeneration as well as for growth and repair of injured tissues, the same process is currently believed to also fuel inflammatory and fibro-proliferative diseases as well as malignancies in different organs, including chronically injured liver. Pertinent to this review, literature data provided in the last decade have unequivocally linked angiogenesis to liver fibrogenesis and CLDs progression, suggesting that angiogenesis may favor fibrogenesis [81,82]. The presence of hypoxic areas within liver parenchyma in the scenario of a developing CLD is the most obvious (but not the only one) stimulus able to switch on the transcription of pro-angiogenic genes through the action of hypoxia inducible factors or HIFs [79,80]. The involvement of angiogenesis in the progression of CLDs, a condition in which a major general mechanism is represented by chronic activation of wound healing, is not really surprising. In addition, the progressive increase of tissue hypoxia ~~which is~~ detected in the CLD scenario is strictly linked to the histopathological modifications of liver tissue in which the increased deposition of ECM components and formation of fibrotic septa, paralleled by vascular changes, with the time lead to an impairment of the oxygen diffusion and consequent up-regulation of pro-angiogenic pathways [81,82].

Evidence linking hypoxia and angiogenesis to liver fibrogenesis is now overwhelming and major issues and considerations can be briefly summarized as follows: i) angiogenesis and fibrogenesis have been shown to develop in parallel in human patients, whatever the etiology, as well as in any experimental model of CLD, as indicated by the presence of a high number of endothelial cells and microvascular structures particularly in the portal tracts and, more generally, within fibrotic septa [81-83]; ii) experimental and clinical studies also revealed that the expression of VEGF, particularly VEGF-A, is usually detected in hypoxic areas of chronically injured liver, with fibrogenic progression being intrinsically associated with a progressive increase in hypoxic areas in

liver parenchyma; iii) in vivo evidence also strongly indicated that VEGF over-expression is strictly associated with hypoxic areas and is mostly limited to hepatocytes as well as to activated HSC/MFs [81,82,84]; MFs are found at close contact with HIF-2α-positive hepatocytes and then are likely to be affected in their behaviour by pro-angiogenic cytokines released by hypoxic hepatocytes. At the same time, MFs themselves are a source of VEGF-A and angiopoietin 1 and express related receptors (VEGFR2, Tie-2) in CLDs [83]; iv) experiments that have compared fibrogenesis progression in HIF-1α liver conditional knock-out mice and related wild type animals, revealed that hypoxia and HIF-1α expression precede fibrosis and that the liver specific silencing of HIF-1α resulted in a significant reduction of liver fibrosis [85]; v) experimental anti-angiogenic therapy, whatever the specific drug or therapeutic strategy employed, resulted in a significant inhibition of fibrogenic progression, being also effective in reducing inflammatory infiltrate, the number of α-SMA positive MFs as well as the increase in portal pressure and, with some drugs, to reduce also formation of porto-systemic collateral vessels and splanchnic vascularization in models of portal hypertensive animals or in cirrhotic animals (reviewed in [81,82,84]).

Experimental studies and a number of studies performed on liver specimens obtained from patients affected by a form of CLDs have also pointed out that hepatic MFs, in particular HSC/MFs, in CLDs are likely to represent a hypoxia-sensitive and cyto- and chemokine-modulated cellular crossroad between critical features like necro-inflammation, pathological angiogenesis and fibrogenesis [81,82,84]. Exhaustive literature has indeed characterized the hypoxia-related pro-fibrogenic and pro-inflammatory role of these cells and major concepts related to the role of hepatic MFs in CLDs angiogenesis may be presented according to their dependence (or not) on hypoxia-related mechanisms.

MFs and their pro-angiogenic role: hypoxia-related mechanisms. Hepatic MFs, in particular HSC/MFs, can efficiently respond to conditions of hypoxia that are progressively increasing in chronically injured liver during CLD progression [81,82,84]. Major concepts can be summarized as follows: i) HSC and HSC/MFs respond to hypoxia in a HIF-1α related way by up-regulating expression of VEGF, Angiopoietin 1 as well as of their related receptor VEGFR-2 and Tie2 [83,86], then behaving as pro-angiogenic cells; ii) HSC/MFs and, likely, all hepatic MFs (including those originating from bone-marrow derived mesenchymal stem cells recruited in chronically injured liver) [4] represent an effective target for VEGF and angiopoietin 1; in these cells VEGF can stimulate proliferation and increased deposition of extracellular matrix components [81,82]), as

well as increased migration and chemotaxis [83,87], the latter response being also significantly elicited in cells just exposed to hypoxia [87]. In particular, oriented migration of MFs in response to either hypoxia or VEGF (as other chemotactic peptides) has been described as a biphasic mechanism that is first switched on by ROS released by either mitochondria or through ligand-receptor related activation of NADPH-oxidase and proceeds through redox-dependent activation of Ras/ERK and c-Jun-NH2-terminal kinase isoforms (JNKs). This is followed by a delayed and sustained phase of migration depending on HIF-1α-mediated, ROS-stabilized, up-regulation of VEGF expression, resulting in the subsequent chemotactic action of extracellularly released VEGF [87]. Immunohistochemistry analysis performed on either human or rodent fibrotic/cirrhotic liver has showed that positive staining for both HIF-2α and heme-oxygenase 1 (HO1, a redox-related marker) is mainly evident in MF-like cells in developing septa and at the border of more mature and larger fibrotic septa suggesting that the scenario is likely to operate also in vivo [87]. The relevant point is that such a morphological evidence overlaps with those observed in human and rat fibrotic/cirrhotic livers [83] where α-SMA positive hepatic MFs expressing VEGF, Ang-1 or the related receptors VEGFR-2 and Tie-2, were detected at the leading edge of tiny and incomplete developing septa, but not in larger bridging septa. Such a distribution may reflect the existence of two distinct phases of the angiogenic process during CLDs [83]: a) an early phase, occurring in developing septa, in which fibrogenesis and angiogenesis may be driven/modulated by HSC/MFs; b) a later phase, occurring in larger and more mature fibrotic septa, in which pro-angiogenic factors and receptors are expressed only by endothelial cells, a scenario that is likely to favor the stabilization of the newly formed vessels.

A very elegant and recent study has provided further evidence for relationships between HIFs and hepatic fibrogenesis by employing hepatocyte-specific von Hippel Lindau protein (VHL) and HIF-1α or HIF- 2α mouse mutants [88]. When mice with liver conditional disruption of VHL (and then with an increased expression of both HIF-1α and HIF-2α) were treated for 2 weeks with an ethanol-containing diet, they developed increased fibrosis. Interestingly, this increase was prevented when simultaneous deletion of HIF-2α (but not HIF-1α), was carried out, suggesting that HIF-2α may prevail in regulating hepatic fibrogenesis [88].

<u>MFs and their pro-angiogenic role: hypoxia-independent mechanisms.</u> Accumulating evidence suggests that hepatic MFs may operate their pro-angiogenic role in a hypoxia-independent manner by responding to a number of stimuli, including pro-fibrogenic polypeptide mediators like

mainly PDGF and leptin. Whether PDGF is concerned, this factor can promote an angiogenic phenotype of HSC that regulates HSC-driven vascular tube formation in vitro and enhanced coverage of sinusoids in vivo, with PDGF then contributing to the modulation of the critical role of HSC/MFs in the modulation of microvascular structure and function in liver parenchyma [89]. A pro-angiogenic action has been proposed also for leptin, which is known to exert pro-fibrogenic effect in the development from NAFLD to NASH [1-5]. Leptin can directly up-regulate in human HSC/MFs the expression of VEGF and Ang-1 as well as of monocyte-chemoattractant protein 1 (MCP-1 or CCL2) [86]. Interestingly, leptin operated the pro-angiogenic actions by recruitment/stabilization of HIF-1α and nuclear translocation of HIF-1 and in vivo the specific leptin receptor ObR co-localized with VEGF and α-SMA after induction of fibrosis in rats. More recently, a study performed on human HSC/MFs has revealed that leptin and PDGF-BB can directly up-regulate VEGF and then the pro-angiogenic role of these cells by a common mechanism involving both activation of the mammalian target of rapamycin (mTOR) pathway as well as generation of ROS via NADPH-oxidase, the latter being relevant for HIF-1α stabilization but not for mTOR activation [90].

PDGF has been shown to exert an additional pro-angiogenic mechanism, that may have a role in vascular remodeling in cirrhosis. An elegant study has shown that cholangiocytes and HSC/MFs in response to PDGF can produce and then release Hedgehog (Hh) ligands contained in microparticles and that this event is likely to be relevant under condition of experimental biliary cirrhosis (BDL model). As previously mentioned, the action of the low amount of Hh ligand released by rare immature ductular-type progenitors is counteracted in normal conditions by expression of Hip expressed by either quiescent HSC or fenestrated SEC. However, under conditions of chronic liver injury HIP expression is repressed and activation of ductular-type progenitor cells may result in PDGF-BB up-regulation and release; this, in turn, can lead HSC/MFs and ductular cells to produce Hh ligands which, in addition to other actions, may also affect SEC gene expression resulting in capillarization of sinusoids and in the release of nitric oxide, then contributing to vascular remodeling in cirrhosis [91].

Autophagy and liver fibrogenesis

Autophagy has recently emerged as an additional mechanism able to fuel fibrogenesis sustained by mesenchymal cells in the liver as well as in other organs [92]. Indeed, autophagy is a catabolic pathway, essential to maintain cellular homeostasis, designed in order to control auto-

degradation of cellular components included in structures defined as autophagosomes. Autophagosome, in turn, fuse with lysosomes leading eventually to the degradation by lysosomal hydrolases of material within these “autolysosomes” and to the release of resultant small molecules back to the cytoplasm. Autophagy is a complex, evolutionary conserved, ubiquitous and strictly controlled pathway that under physiological conditions works at very low levels of activity but may be rapidly induced by several conditions of cellular stress. Formation of autophagosomes and regulation of autophagic machinery requires the involvement of 14 genes defined as autophagy-related genes (Atg) and proceeds through distinct essential steps termed induction, cargo recognition/selection, vesicle and autophagosome formation, fusion and breakdown [95].

Induction. Autophagy induction is physiologically repressed by the serine/threonine protein kinase mTOR (mammalian target of rapamycin), which inactivates the components that are necessary to switch on the pathway. Any condition (starvation and different types of cellular stress) resulting in inactivation/inhibition of mTOR signaling will lead to phosphorylation and activation of the complex between Atg13 and focal adhesion kinase family-interacting protein of 200 kD (FIP200) by Unc-51-like kinases 1 (ULK1) and 2 (ULK2), resulting in activation of autophagy pathway and the initial formation of autophagosomes with membranes that are believed to originate from plasma-membrane, mitochondria and endoplasmic reticulum [94].

Cargo recognition/selection. Autophagy machinery recognizes the “cargo” to be degraded through the cytosolic adaptor protein P62/sequestosome 1 that binds to the microtubule-associated protein 1 light chain 3 (LC3) in order to deliver the cargo for autophagic degradation [93,94].

Autophagosome formation. It requires the involvement of class III phosphatidylinositol 3-kinase (PI3K) complex, which also include the PI3K vacuolar protein sorting 34 (Vps34), beclin1, Atg 14 and p150, with beclin 1 being inactivated in normal conditions through its binding to the anti-apoptotic protein Bcl2 (B-cell lymphoma/leukemia-2) [93,94]. The PI3K complex then recruits two ubiquitin-like conjugation complexes formed by several Atg proteins, one needed for autophagosome formation and the second for its elongation, both involving a key player like Atg7. A very complex series of reactions, leads finally to the binding of these complexes to the nascent phagophore and then the formation of autophagosomes [93,94].

Fusion and breakdown. In the previous step, LC3 precursor is cleaved by Atg4 and converted to LC3-I which, in turn is conjugated to phosphatidyl-ethanolamine by Atg7 and Atg3, forming then LC3-II that remains on the autophagosome membrane until its fusion with the lysosomes [96].

Fusion of autophagosomes with lysosomes is then followed by the degradation of the sequestered materials by lysosomal hydrolases [97].

Whether fibrogenesis is specifically concerned, high levels of autophagy have been detected in pro-fibrogenic cells in any tissue investigated so far, including dermal and cardiac fibroblasts, hepatic stellate cells, mesangial cells and synovial fibroblasts [92]. Data relating autophagy to liver fibrogenesis first emerged independently by two different groups that showed evidence for an increase of autophagy in activated HSC [98,99], both suggesting that autophagy has a first role in the loss of perinuclear lipid droplets (containg retinyl esters) that accompanies trans-differentiation of quiescent HSC towards the activated phenotype. In a first study autophagy was detected during in vitro activation of primary mouse HSCs by investigating typical autophagy-related markers, including LC3. Moreover, inhibition of the autophagic process using bafilomycin A1 resulted in a significant decreased proliferation and expression of activation markers in either mouse or human activated HSCs [99]. A second study went much further by describing for the first time in HSC isolated from murine fibrotic livers (models of liver fibrosis induced by either CCl_4 or thioacetamide) the involvement of autophagy during the process of culture activation by analyzing markers like P62, LC3I and LC3II [98]. Similar findings were also described in the same study in HSC in human specimens from patients chronically infected by HBV. Moreover, in culture activated mouse HSCs the inhibition of autophagy, obtained by either employing the pharmacological agents such as 3-methyl-adenine and chloroquine or by specifically knocking down Atg7 or Atg5 expression, resulted in a significant reduction of expression of critical genes involved in fibrogenesis and encoding for collagen α1(I), collagen α2(I), PDGF-RB, α-SMA and MMP2 [98]. Even more convincing have been results obtained in a specifically generated transgenic mouse line in which expression of Atg7 was specifically attenuated in HSC within the liver by crossing $Atg7^{loxP/loxP}$ mice with mice expressing cre recombinase under the glial fibrillary acidic protein (GFAP) promoter (GFAP-cre mice). Using this in vivo approach Authors showed that following chronic liver injury induced by either CCl_4 or thioacetamide the number of HSCs was not altered and liver fibrosis was almost completely abolished when compared to proper control mice [98]. Interestingly, in this in vivo conditions the inhibition of autophagy resulted not only in the prevention of activation of HSC but also in the preservation of perinuclear lipid droplets (mainly containing retinyl esters) which are typically lost by activated HSC during the activation/trans-differentiation process. Authors of this study, hypothesized that in HSC the metabolism of lipid

droplets may provide cellular energy critical for fueling catabolic pathways of cellular activation, a feature which indeed disappears when inhibiting autophagy in these cells.

In a more recent study, the same research group has provided further experimental evidence that correlates endoplasmic reticulum (ER) stress and generation of ROS to autophagy and HSCs activation [100]. The involvement of ER stress was hypothesized on the basis of the postulate that any condition that augments protein synthesis is likely to increase demands on the endoplasmic reticulum (ER) folding capacity, then potentially triggering the classic unfolded protein response (UPR) in order to cope with resulting ER stress. Moreover, generation of ROS is known to represent a common feature of hepatic fibrogenesis, and crosstalk between altered redox homeostasis and ER stress has been proposed [1-5,18]. By exposing cultured HSCs to ROS or by feeding ethanol in vivo, Authors were able to detect up-regulation of UPR and, in particular, of the IRE1α–related pathway, as well as of the Nrf2 antioxidant response. Interestingly, in both conditions the IREα-related splicing of XBP1 mRNA was found to trigger autophagy. Moreover, blockade of the IRE1α pathway in HSC significantly decreased both their activation and autophagic activity in a p38 MAPK-dependent manner, leading to a reduced fibrogenic response.

The overall scenario emerging from these recent findings suggests then that the activation of autophagy may provide the energy required for initiating and perpetuating the activated, MF-like phenotype of HSC in the progression of CLD.

Inflammasomes and liver fibrogenesis

As already mentioned in previous sections, inflammatory response has a major role in sustaining fibrogenesis and then the progression of CLDs, whatever the etiology. In particular, resident cells of innate immunity, through the binding to their pattern recognition receptors (PRRs), can either detect pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) from injured cells, the latter event leading to the so-called sterile inflammation [101]. Several DAMPs can be released or generated into the extracellular environment by dying cells or in relation to abnormal metabolism, and then trigger sterile inflammation, including proteins like: i) high-mobility group box 1 (HMGB1) [102], ii) heat shock proteins (HSPs) [103], iii) proteins of extracellular matrix generated following tissue injury (i.e., hyaluronan, heparin sulfate and biglycan) as modified/cleaved by enzymes released from dying cells or by other proteases [104,105]. Non-protein DAMPs such as ATP and uric acid ~~(that)~~ can be released or generated during cell injury and death [106]; mitochondria are a rich source of DAMPs

like, in addition to ATP, mitochondrial DNA, formyl peptides and cytochrome C [107] which are effective stimulators of inflammation. Along these lines, inflammasomes represent a group of protein complexes able to recognize PAMPs and DAMPs and that control activation of the proteolytic enzyme caspase-1, which in turn regulates maturation of the pro-inflammatory cytokines IL-1β and IL-18 [108,109]. The release of IL-1β and IL-18 can result in the activation of an acute inflammatory response, resulting in the production of TNF-α, interferon-γ (IFN-γ) as well as chemotaxis of immune cells, and induction of tissue injury. In order to obtain full activation (that is, to obtain the effective synthesis and release of active IL-1β and IL-18) the inflammasome typically requires two distinct signals: i) a first priming signal usually triggered either by PAMPs interacting with PRRs as well as by inflammatory citokines (TNF or IL-1) interacting with the related receptor; the first signal is designed to activate transcription of the genes encoding pro-IL-1β, pro-IL-18 and NLRP3 [110]; ii) the second signal can be provided by various inflammasome ligands (including PAMPs) through involvement/activation of cytosolic NLR (NOD-like receptor) proteins, particularly NLRP3, which in turn leads to activation of caspase-1 and cleavage of pro-IL-1β and pro-IL-18 into the active forms [111]; the second signal can be reinforced by ROS generated after exposure to PAMPs and DAMPs. Inflammasome can be modulated by at least two further events: a) it has been reported that a cross-talk exists between inflammasome and autophagy; in particular, autophagic suppression of ROS generation (i.e., by sequestering in autophagosomes defective mitochondria which may represent a source of ROS) indirectly inhibits inflammasome activity [112]; b) inflammasome activity is inhibited by either cell-cell interactions or secreted factors (for example IFNγ) involving lymphocytes of adaptive immunity, particularly CD4+ effector and memory T cells [113].

Pertinent to this review is the emerging evidence suggesting that this theoretically acute inflammatory pathway seems to be critical also for sustaining fibrogenesis in several organs including the lung, liver, and skin [114]. Whether CLDs are concerned, recent experimental studies, performed by means of selective depletion of macrophages, have outlined that macrophage activation in liver fibrosis has at least two distinct phases: a) during fibrogenic progression of CLDs liver macrophages contribute to the deposition of ECM components matrix; b) during fibrosis reversion macrophages contribute mainly to matrix degradation [115]. Interestingly, mice lacking inflammasome components have a reduced rate of fibrosis, a finding which indicates that the inflammasome may be critical at least in the initial phase of macrophage-related ECM deposition. However, it should be emphasized that the old postulate of IL-1β as a cytokine produced only by

activated leukocytes and dendritic cells has been challenged by recent data which unequivocally indicate that other cells can express inflammasome components [115], including immune cells as well as hepatic stellate cells and hepatocytes [116,117]. Along these lines, we know now that natural killer (NK) and NK-T cells, T cells with Th1, Th2, Th17 and Th22 skewing as well as dendritic cells (DC, particularly in NASH) have a relevant role in liver fibrosis [118], although we still miss clear evidence of whether inflammasome in these immune-related cells is involved in fibrogenesis.

At present, literature data concerning the involvement of inflammasome activation in the progression of CLDs are available for alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH).

In alcoholic liver disease (ALD), Toll like receptors (TLRs) and mainly TLR4 have been reported to be involved in tissue damage as shown by employing TLR4 deficient mice [119], with a relevant role likely played by PAMPs since significant reduction in the bacterial load of the intestine resulted in a significant improvement of liver injury [120]. At present, just a single study has investigated specifically the role of inflammasome components in a rodent model of ethanol-mediated liver injury [121]. In this study Authors employed mice treated with a IL-1R antagonist (IL-1Ra) as well as 3 mouse models deficient in regulators of IL-1β activation (Caspase-1 and the adaptive protein ASC) or signaling (IL-1R1) and found that IL-1β signaling was required for the development of alcohol-induced liver steatosis, inflammation, and injury. Moreover, the use of bone marrow chimeras revealed that increased levels of IL-1β were due to up-regulation of Caspase-1 activity and inflammasome activation in bone marrow-derived Kupffer cells rather than in hepatocytes. According to these findings, experimental administration of IL-1Ra, which blocked IL-1 signaling, resulted in a significant decrease in ethanol-induced steatosis, inflammation and liver injury. Although fibrosis was not directly investigated, Authors also reported additional positive events following IL-Ra administration including: i) reduction of expression of TGF-β1 and procollagen 1α1 and of serum levels of hyaluronic acid, TIMP-1 and N-terminal peptide of procollagen type III (PIIINP); ii) an overall reduction in the progression of ethanol-mediated liver injury; iii) a more rapid improvement after stopping ethanol administration [121].

Whether NASH is concerned, data are mainly coming from experimental models in which a major problem is represented by the low level of liver injury that can be detected and in the difficulty to detect then DAMPs. However, similar to what reported for ASH, also in experimental NASH and in human NASH patients a clear increase in LPS levels has been detected, which is likely

to be due to intestinal bypass [122]. Although the role of fatty acids in activating TLRs is still controversial, although described, interesting studies on transgenic mice have shown that the absence of either TLR4 or TLR9 results in a significant reduction of NASH related parameters like histological score, hepatocyte death (apoptosis and necrosis) and markers of fibrogenesis [123-125]. These studies pointed out that TLR9 seems to be relevant for significant IL-1β production from Kupffer cells in experimental NASH not only in relation to steatosis but also in relation to HSC activation and hepatocyte death. In particular, IL-1β (which is not able to induced cell death by itself) may lead to liver injury and hepatocyte death indirectly by sensitizing hepatocytes to more conventional death signals like TNF-α. [123,126].

A relevant point in relation to the emerging pro-fibrogenic effects of inflammasome activation is to understand whether this is somewhat related to a direct effect on hepatic MFs and their precursors exposed to DAMPs or is more indirect, being caused by IL-1β and IL-18 released by DAMPs-activated cells of innate immunity. The still scarce available literature data seem, at present, to possibly support both hypotheses. Indeed HSC express the critical inflammasome components and positively respond to classic DAMPs like uric acid crystals, which are known to activate NLRP3 inflammasome. Although uric acid crystals may represent somewhat "alien" DAMPs for HSC, exposure of either human or mouse HSC to these crystals results in a further change in morphology (more fibroblastoid phenotype) as well as in an increased expression of TGF-β and collagen type I, changes which do not occur in HSC lacking the adaptive protein Asc [114].

Concerning the "indirect" pathway, indeed Kupffer cells respond to stimuli able to activate NLRP3 inflammasome by producing and releasing IL-1β and IL-18 [127]. The question is whether IL-1β, which is known to increase during experimental liver fibrosis, may be considered as a pro-fibrogenic cytokine. The few data available at the moment seems to suggest that this may be in some way true, since liver fibrosis is significantly lower in IL-1R deficient mice [124]. In wild type mice experimental chronic liver injury resulted in up-regulation of IL-1, MMP-9 and MMP-13 levels that were elevated before the onset of HSC activation and liver fibrogenesis, whereas IL-1 receptor-deficient mice exhibited less liver damage and reduced fibrogenesis. Moreover, advanced fibrosis, as determined by type-I and -III collagen mRNA expression and fibrotic septa, was partially attenuated by the deficiency of IL-1. Authors also report that in the early phase of liver injury, expression of MMP-9, MMP-13 and TIMP-1 correlated nicely with IL-1 levels, with

MMP-9 being expressed by desmin-positive cells (i.e., HSC). Accordingly, MMP-9-deficient mice were partially protected from liver injury and HSC activation [127].

Another potentially interesting experimental study related to the role of IL-1β was performed using the choline-devoid and aminoacid-refined (CDAA) diet in order to induce NASH in wild type mice and in TLR9(-/-) mice. These transgenic mice where characterized by less steatohepatitis and liver fibrosis than WT mice as well as by suppression of IL-1β production by Kupffer cells. Of interest, IL-1β induced fibrogenic responses in HSCs, including secretion of tissue inhibitor of metalloproteinase-1 whereas IL-1R(-/-) mice had reduced steatohepatitis and fibrosis, compared with WT mice. Mice deficient in MyD88, an adaptor molecule for TLR9 and IL-1R signaling, also had reduced steatohepatitis and fibrosis [123].

Natural killer and natural killer T cells in liver fibrogenesis: friends or foes ?

In recent years, the role of NK and NKT cells in liver fibrogenesis has received great attention because these cells are enriched among liver lymphocytes and are also markedly altered in various liver diseases of different etiology [128]. Of interest, NK cells can prevent liver fibrosis whereas NKT cells can either inhibit or favor excess ECM deposition.

NK cells are lymphocytes of the innate immune system which are able to recognize and kill infected and tumorigenic cells, representing a lineage of lymphoid cells that, differently from T and B cells, do not express an antigen receptor. NK cells use an array of cell surface receptors to detect changes in the expression of host cell surface molecules that typically appear on either viral-infected, transformed or injured cells [129]. NK do not kill normal host cells because of inhibitory receptor molecules like killer Ig-like receptors (KIRs) and Ly-49A and CD94/NKG2 receptors that recognize MHC class I molecules expressed on most normal cells. By contrast, NK cells kill target cells through a number of stimulatory receptors (NKp46, NKp30, NKp44, NKG2D, and DNAX accessory molecule-1 or CD226) [128,129] able to recognize specific ligand on target cells, resulting in NK activation followed by cell killing by either exocytosis of perforin- and granzyme granules or by using FAS ligand, TNF-α, and TNF-related apoptosis-inducing ligands (TRAIL).

NKT cells are instead a heterogeneous group of T lymphocytes that recognize lipid antigens presented by the non-classical MHC class I-like molecule CD1 [130]. Human tissues express five distinct isoforms of CD1, including CD1a, -b, -c, -d, and -e. Liver NKT cells are mostly CD1d-dependent NKT defined as type I NKT cells or classical or invariant NKT (iNKT) cells because they

express an invariant T cell receptor α (TCR-α) chain. iNKT activation by lipid antigens such as α-galactosylceramide is followed by release of significant levels of IFN-γ, IL-4, IL-13, TNF-α, IL-17, and many other cytokines as well as by production of perforin, FasL and TRAIL in order to kill target cells.

Hepatic NK cells as inhibitors of fibrogenesis. NK cells are located in the hepatic sinusoids close to liver non-parenchymal cells and represent a unique organ-associated NK cell population, with a rapid turn-over and continuously substituted by bone marrow-derived cells. NK cells account for approx. 30-50% of lymphocytes in human liver and their number greatly increases in pathological conditions (mainly related to viral infection or acute and chronic inflammation) [128]. What is remarkable for liver NK cells is that they can selectively kill early activated HSC but not quiescent or fully activated and MF-like cells, as shown by several laboratories in culture experiments (i.e., co-culture of HSC and NK cells) but also in animal models and, even more relevant, in human patients [128]. Such a selective killing is related to the fact that during the process of activation early activated HSCs produce retinoic acid, which upregulates the NK cell activating ligand retinoic acid inducible gene 1 (RAE1) expression on HSCs. RAE1 can bind to NKG2D on NK cells then leading to NK cell activation and HSC death through TRAIL- and NKG2Ddependent mechanisms [131]. This is facilitated by the fact that early activated HSC also express on their surface TRAIL receptors. Fully activated and MF-like cells have lost their stores of retinol and do not produce RA and RAE1, becoming then resistant to NK cells. In addition, activated NK cells also produce large amount of IFN-γ, which can induce HSC apoptosis and cell cycle arrest, as well as by enhancing NK cell killing of early activated HSC [131,132]. In addition, NK cells can selectively kill senescent activated HSC, which are cell cycle arrested cells that accumulate in fibrotic septa during chronic liver injury and become inefficient in ECM synthesis but able to up-regulate expression of extracellular matrix-degrading enzymes [133, 134]. It has been proposed that senescence-activated HSCs express elevated levels of NK cell-activating ligands, becoming then sensitive to NK cell killing [133].

NKT cells as a two-edged sword in liver fibrogenesis. NKT cells have a more complex role in the pathogenesis of liver fibrosis likely because there are more types of NKT cells, with type I and type II playing different and sometimes opposing functions in the liver [128]. Moreover, as nicely recently reviewed [128] we essentially ignore the mechanisms by which NKT cells are activated in vivo by endogenous ligands and cytokines, with NKT cells that, after activation, become tolerant and nonresponsive to subsequent stimulation. Moreover, activated NKT cells can release high

amount of either anti-fibrotic (e.g., IFN-γ) and pro-fibrotic (e.g., IL-4, IL-13, hedgehog ligands, and osteopontin) cytokines, together with many other cytokines, chemokines, and mediators that can differentially regulate liver fibrogenesis. The role of NKT cells has been extensively studied in either experimental models and in human patients which often provided contrasting or even opposite conceptual results. Clinical studies performed in viral hepatitis patients (HCV etiology) suggest that activated human HSCs can stimulate human NK cell activation which, in turn, can kill activated human HSCs and then inhibit liver fibrosis. Moreover, IFN-α treatment has been shown to increase the ability of human NK cells from HCV patients to kill activated HSCs [135,136]. However, the scenario may change depending on the stage of CLD progression since NKT cells from HCV patients without cirrhosis produce both anti-fibrotic (IFN-γ) and pro-fibrotic (IL-4 and IL-13) cytokines, whereas NKT cells from cirrhotic HCV patients preferentially produce IL-4 and IL-13. The overall feeling is that NKT cells can promote liver fibrogenesis in HCV patients [137] as possibly also in NASH patients [138]. However, in patients affected by ALD the scenario is opposite, with chronic consumption ethanol reported to suppress activation of NKT cells, then facilitating fibrogenesis and then fibrosis. In a recent exhaustive review Gao and Radaeva [128] have proposed a reasonable model to interpretate these contrasting results. The hypothesis relies on the knowledge that NKT cells can be activated by either lipid antigens (such as α-GalCer) presented by CD1d on antigen presenting cells (APCs) as well as by several cytokines, including mainly IL-12, IL-18, IFNα and IFNβ. The critical point is that once activated NKT cells can rapidly produce a wide variety of anti-fibrotic and pro-fibrotic mediators that are potentially able to either down-modulate or to enhance liver fibrosis. The resulting effect is then related to the balance between these mediators and published data suggest the following concepts: i) liver fibrogenesis is stimulated when activated NKT cells mainly produce mediators like IL-4, IL-13, osteopontin or Hh ligands, with an overall meaning of potentiating activation and proliferation of HSCs; ii) liver fibrogenesis is inhibited when NKT cells mainly produce IFNγ, perforin or FasL, which are of course relevant to induce HSC apoptosis and cell cycle arrest.

Serotonin signaling in liver fibrogenesis

Serotonin or 5-hydroxy-triptamine (5-HT) is a very old molecule from an evolutionary point of view that has a role in normal wound healing in different organ and tissues but, as recently emerged, in conditions of chronic injury can promote aberrant wound healing resulting in tissue fibrosis and impaired organ regeneration [139]. 5-HT in humans acts as a neurotransmitter in the central nervous system but it is also produced by enterochromaffin cells of the gut with a role in

controlling smooth muscle contraction. 5-HT once in the plasma can be taken up through a specific serotonin transporter (SERT) by either platelets and mast-cells, to be then released following activation of these cells resulting in a number of actions including mainly vasoconstriction/vasodilation, cardiac development and function, metabolic rate and temperature control [140]. 5-HT can exert its actions on target cells expressing one of the several specific receptors that are the products of genes encoding for G-protein coupled seven-transmembrane receptors (GPCRs) and classified as either 5-HT1 (1A, 1B, etc) or 5-HT2 (2A, 2B, etc) receptors, with an additional 5-HT3 receptor represented by a ligand-gated ion channel [141]. What makes serotonin able to exert a wide range of actions on different target cells is indeed related to the specific G protein to which 5-HT receptors are coupled. In particular, 5-HT receptors have been described to couple with either Gαq/11, Gαi/o or Gαs, each of which can trigger distinct downstream signaling events: i) Gαq coupled receptors can signal through two distinct pathways, one operating through diacylglycerol (DAG) and inositol phosphates, protein kinase C (PKC) activation and increased cytosolic Ca2+ levels, the other involving Rho activation and downstream signaling leading to stress fibers and focal adhesions formation, affecting cell migration and adhesion; ii) in neurons Gαi/o coupled receptors operates by suppressing adenylate cyclase and cAMP levels [141]; iii) Gαs coupled 5-HT receptors transduce signals by activating adenylate cyclase and then through cAMP, protein kinase A (PKA) and cAMP response element binding protein (CREB) [141].

Serotonin, as a molecule involved in normal wound healing, can either stimulate vasoconstriction or vasodilation depending on the specific tissue in which is released (in the liver, 5-HT is believed to promote vasoconstriction of liver sinusoids) depending likely to the specific 5-HT receptors which are expressed on vascular endothelial and smooth muscle cells [142]. More relevant, serotonin contributes apparently to leukocyte recruitment and retention at the site of injury and has been reported to affect behavior and function of cells of innate and adaptive immunity. For example, human monocytes primed by LPS through binding to TLR4 respond to 5-HT by increasing their secretion of critical pro-inflammatory peptides, including IL-1β, IL-6, IL-8, IL12p40 and TNF-α [139]. In addition, 5-HT has also reported to inhibit monocyte apoptosis via the 5-HT1 and 5-HT7 receptors allowing monocytes to remain in tissues and to promote inflammation [143]. Other cells like eosinophils and mast cells respond to 5-HT by migrating whereas dendritic cells respond by up-regulating expression of IL-6 [139,144]. The response of T lymphocytes to 5-HT is rather a matter of concentration with low levels of serotonin stimulating proliferation and IL-2

expression [144] and high levels acting as inhibitory signals [145]. Interestingly, mice which lack peripheral serotonin (Tph−/− mice) have been reported to be less susceptible to steatosis-induced hepatic inflammation, with 5-HT acting in a pro-inflammatory way by eliciting intracellular ROS generation in hepatocytes following 5-HT uptake and degradation, an event which is favored by IL-1β- and TNF-α-dependent increased expression of the mentioned SERT transporter [146].

Whether liver parenchyma is specifically concerned, a number of recent studies have revealed the involvement of serotonin signaling in either regeneration (i.e., restoration of hepatic mass) and fibrogenesis. By using the conventional procedure of 70% partial hepatectomy (PHX) as a model to induce liver regeneration it has been described that, following surgical intervention, serotonin is rapidly mobilized from gut and accumulates in the remnant liver, possibly delivered here by platelets [148], although the latter hypothesis has been recently challenged. Available data suggest in any case that 5-HT can sustain hepatocyte proliferation following PHX, likely by acting through a restrict number of receptors (5-HT1A, 2A and 2B), as also unequivocally indicated by the significant impairment of regeneration observed in Tph−/− mice [147,148]. Recently, it has proposed that cholangiocytes may represent an additional source of serotonin as resident liver cells, with serotonin that once released can in turn represses cholangiocyte proliferation through a negative feedback mechanism [149]. Interestingly, these Authors provided evidence suggesting that under conditions of biliary injury a peculiar cross-talk between cholangiocytes and MFs may occur, with 5-HT triggering TGFβ1 production by MFs (likely portal MFs) which, in turn, can act back on cholangiocytes to repress expression of tryptophan-hydroxylase 2 (TPH2, the enzyme generating serotonin) and enable their proliferation. Although this latter study represents at the same time an indication of the pro-fibrogenic potential of serotonin, it is correct to underline that a more direct involvement of serotonin in fibrogenesis was first suggested by the group of Derek Mann early in 2006 with a paper describing that HSC can strongly up-regulate expression of 5-HT2A and 5-HT2B receptors during the activation/trans-differentiation process towards the activated and MF-like phenotype [150]. In the same study it was also reported that HSC express the SERT transporter and are then able to uptake, release and respond to 5-HT by means of this autocrine route as well as to respond to serotonin released by either platelets or cholangiocytes [150]. Of interest, Authors also reported that the use of 5-HT2 receptor-selective antagonists resulted in inhibition of HSC proliferation and in the induction of apoptosis, a relevant issue from a therapeutic point of view. The same group provided another interesting study that revealed a major role for 5-HT2B receptor on HSC-derived myofibroblasts: signaling through this receptor has

been described to be able to promote fibrogenesis and, at the same time, to contribute to impair regeneration in a diseased liver [151]. Unequivocal evidence was provided in the latter study by using 5-HT2B knockout mice or specific pharmacological antagonists of 5-HT2B, both procedures resulting in stimulation of proliferation and suppression of fibrosis in different experimental models of CLD. The dual function attributed to 5-HT/5-HT2B signaling is not overall surprising since it is likely to rely on the fact that such a pathway can trigger ERK- and JunD dependent activation of TGFβ1 expression, with TGFβ1 representing both a potent suppressor of hepatocyte proliferation and a powerful stimulator of fibrogenic gene expression [139]. As suggested by Mann and Oakley in a recent review [139], at present the overall message concerning the role of serotonin may be synthetized as follows: i) 5-HT/5-HT2A signaling in hepatocytes is able to sustain proliferation (i.e., regeneration) of hepatocytes; ii) 5-HT/5-HT2B signaling in the scenario of fibrogenic progression of CLDs involves and activates HSC to release TGFβ1 which can both sustain fibrogenesis and inhibit hepatocyte proliferation.

Concluding remarks

Liver fibrogenesis is by definition, irrespective of etiology, a dynamic and highly integrated molecular, tissue and cellular process that leads to the progressive accumulation of extracellular matrix (ECM) components in an attempt to limit hepatic damage in a CLD. We know at present that progressive fibrogenesis, sustained by a heterogeneous population of MFs that may originate from different cells sources, has a tremendous clinical impact since it can lead to development of cirrhosis and related complicances, including the development of hepatocellular carcinoma, the most common primary liver cancer. In this review we made an attempt to introduce the reader to the most relevant data and concepts emerged recently from basic and clinical studies which are continuously unravelling molecular, cell and tissue mechanisms having a role in driving fibrogenesis and then CLD progression. All these studies are fundamental in order to offer putative new “targets” for more selective and effective therapeutic strategies to be tested with properly designed clinical trials. These strategies may represent in the future realistic options for a changing scenario in which fibrosis and, possibly, compensated cirrhosis, may be effectively reverted and or at least delayed in their progression, then decreasing the need for liver transplantation (i.e., at present the only effective therapy for end-stage liver disease) .

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Figure Legends.

Figure 1.

Progression of chronic liver diseases: tissue events. A scheme reporting major tissue events involved in the fibrogenic progression of chronic liver disease is offered that links persisting liver injury, irrespective to the etiology, to chronic inflammation and activation of wound repair, with emphasis on the role of hepatic myofibroblasts in driving the disease to significant fibrosis and eventually to cirrhosis, liver failure and hepatocellular carcinoma.

Figure 2.

The "chronic" scenario: a long-standing story of cross-talk between hepatic cell populations. A simplified scheme of the complex scenario of interactions between major hepatic cell populations involved in liver fibrogenesis is offered. For any cell population is also indicated a list of major mediators that once produced and released are able to significantly affect the behavior of neighbouring cells. Hypoxia is also indicated as a condition able to affect responses of hepatic cell populations.

Figure 3.

Origin of hepatic myofibroblasts. The scheme recapitulates actual knowledge on the origin / cell source of pro-fibrogenic hepatic myofibroblasts.

Figure 4.

The fate and role of hepatic myofibroblasts. In the scheme are summarized established or proposed fate and role for hepatic myofibroblasts involved in the progression of a chronic liver disease, irrespective of the etiology or the cellular source of MFs.

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Figure 1

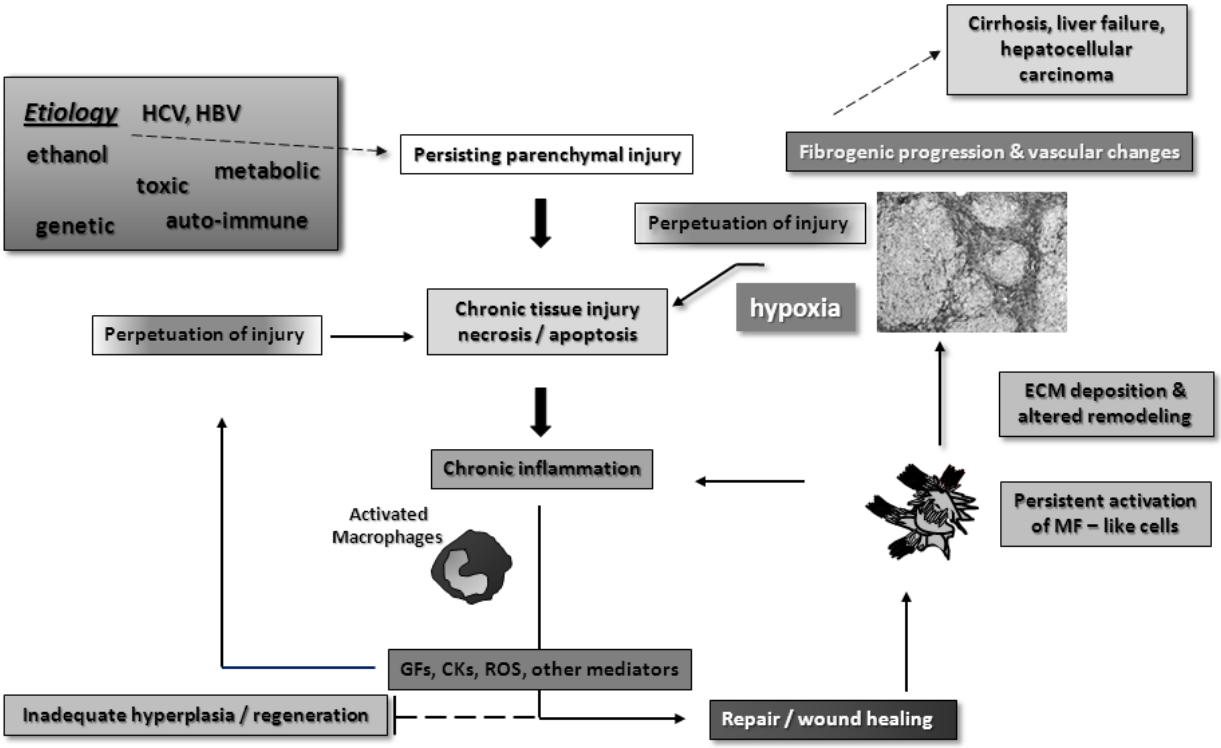


Figure 2

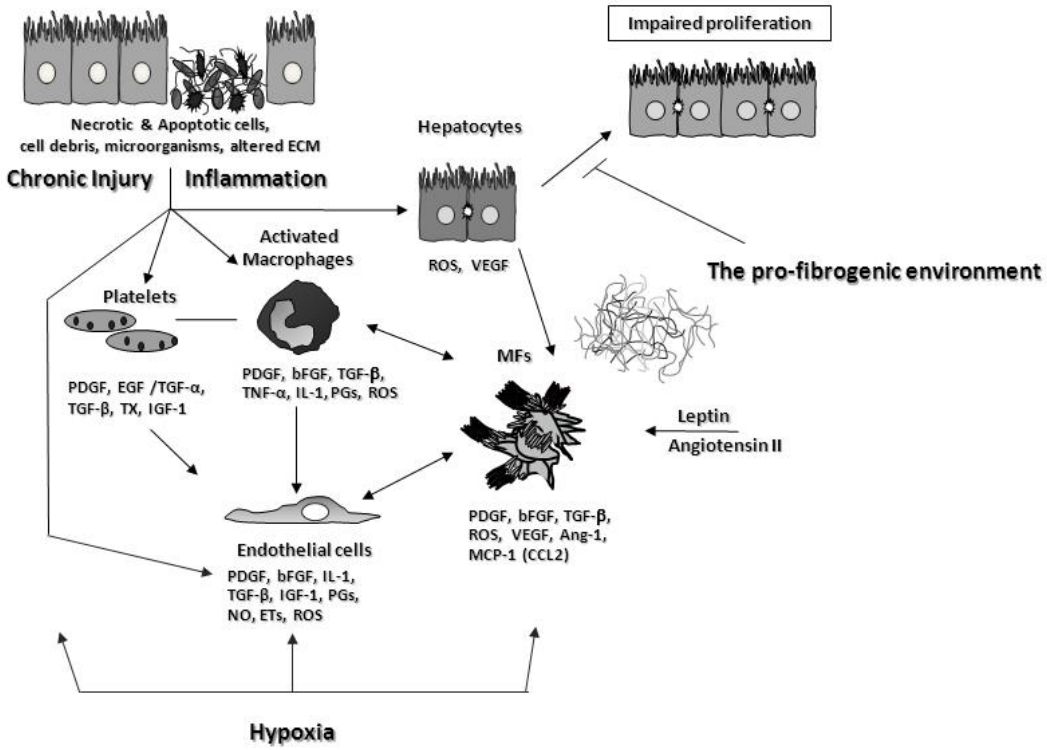
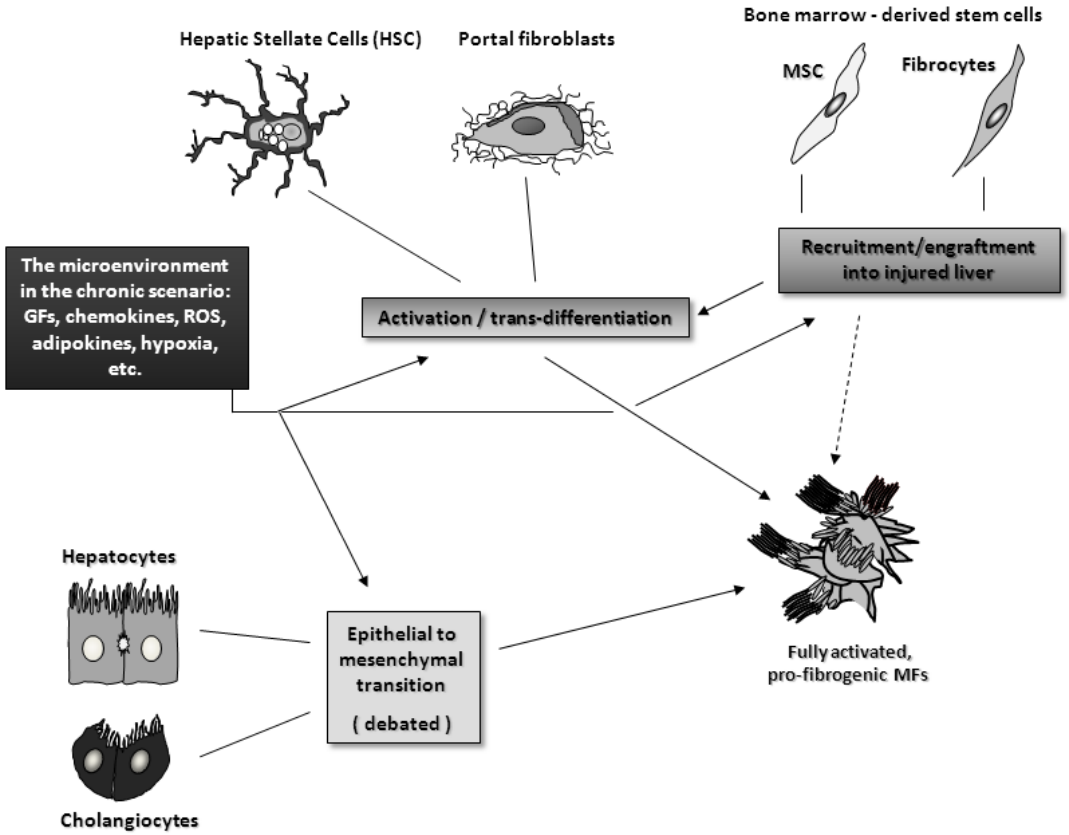

Impaired proliferation
Necrotic & Apoptotic cells,
cell debris, microorganisms, altered ECM
Hepatocytes
Chronic Injury
Inflammation
Activated
Macrophages
ROS, VEGF
The pro-fibrogenic environment
Platelets
MFs
PDGF, EGF /TGF-α,
TGF-β, TX, IGF-1
PDGF, bFGF, TGF-β,
TNF-α, IL-1, PGs, ROS
Leptin
Angiotensin II
PDGF, bFGF, TGF-β,
ROS, VEGF, Ang-1,
MCP-1 (CCL2)
Endothelial cells
PDGF, bFGF, IL-1,
TGF-β, IGF-1, PGs,
NO, ETs, ROS
Hypoxia

Figure 3


Hepatic Stellate Cells (HSC)
Portal fibroblasts
Bone marrow - derived stem cells
MSC
Fibrocytes
Recruitment/engraftment into injured liver
The microenvironment in the chronic scenario: GFs, chemokines, ROS, adipokines, hypoxia, etc.
Activation / trans-differentiation
Hepatocytes
Epithelial to mesenchymal transition (debated)
Fully activated, pro-fibrogenic MFs
Cholangiocytes

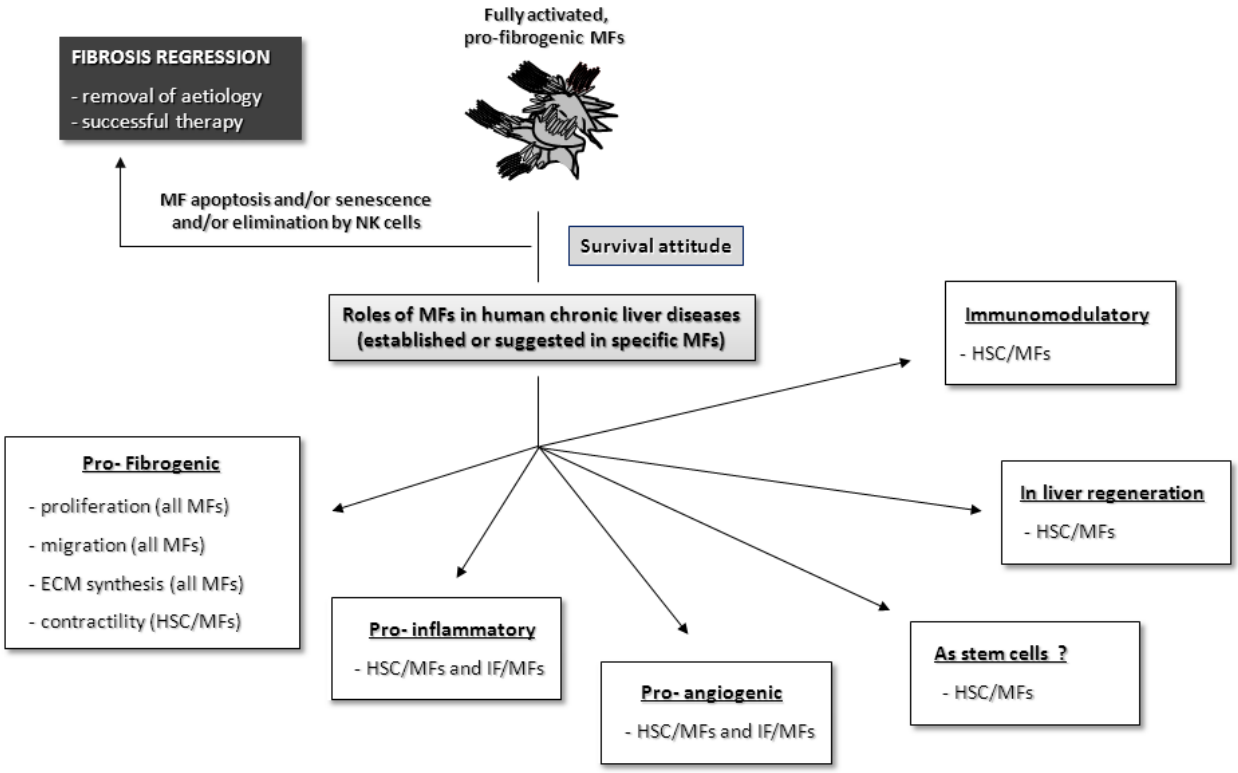

FIBROSIS REGRESSION
- removal of aetiology
- successful therapy
Fully activated,
pro-fibrogenic MFs
MF apoptosis and/or senescence
and/or elimination by NK cells
Survival attitude
Roles of MFs in human chronic liver diseases
(established or suggested in specific MFs)
Immunomodulatory
- HSC/MFs
Pro- Fibrogenic
- proliferation (all MFs)
- migration (all MFs)
- ECM synthesis (all MFs)
- contractility (HSC/MFs)
In liver regeneration
- HSC/MFs
Pro- inflammatory
- HSC/MFs and IF/MFs
Pro- angiogenic
- HSC/MFs and IF/MFs
As stem cells ?
- HSC/MFs

Figure 4